EPIDEMIOLOGY OF *NEOSPORA CANINUM* IN DAIRY CATTLE AND WILD CANIDS ON PRINCE EDWARD ISLAND

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Abstract

The main aim of the studies described in this thesis was to obtain more epidemiological information on *Neospora caninum* and the purported sylvatic life cycle of this parasite.

The significance of *N. caninum* in dairy herds on Prince Edward Island (PEI) is reported in Chapter 2, by testing bulk milk samples. The prevalence of dairy farms with a within-herd seroprevalence ≥15% on PEI was 10.2% in June 2005. In 11 farms that were considered positive based on bulk milk samples, blood samples were collected of all adult cows in September 2005, in conjunction with a fourth bulk milk sample on the same day. The results of this study demonstrate that the prevalence of *N. caninum* in dairy farms can be estimated using a bulk milk enzyme linked immunosorbent assay (ELISA).

Three ELISAs, 2 indirect fluorescent antibody tests (IFATs) and 1 *N. caninum* agglutination test (NAT) were compared using sera of randomly collected dairy cattle from Atlantic Canada. One IFAT performed well (sensitivity and specificity: 0.97 and 0.97) using reference sera, therefore results from this IFAT could subsequently be used as reference standard to calculate test characteristics for the other assays. Sensitivity was ≥0.89 for all assays except the NAT which had a significantly lower sensitivity (0.66). Specificity was high (>0.94) for all assays except for one indirect ELISA (specificity=0.52). The performance characteristics observed for most assays in this study (Chapter 3) make them useful for screening antibodies to *N. caninum* in cattle.

The effect of hemolysis on serological assays was studied in Chapter 4 and demonstrates that an indirect ELISA produced better test characteristics using hemolysed fox sera. A competitive ELISA had the advantage of not being species specific, and performed equally well with non-hemolysed sera from cattle and foxes. However, caution is required when using this competitive ELISA with hemolysed fox sera, because it had a significantly reduced specificity.

Chapter 5 describes the population structure and habitat characteristics of wild canids on PEI. The average age of foxes was 1.5 years (range 0.5-13.5, median 0.5) and for coyotes 2.1 years (range 0.5-13.5, median 1.5). The proportion of juvenile (less than a year of age) foxes and coyotes was 58 and 48%, respectively. The observed sex-ratio was equal, and average litter size for foxes and coyotes was 5.0 (range 0-7) and 5.2 (range 0-11), respectively. The main habitat for foxes was agricultural land and forest was the main habitat for coyotes.

Chapter 6 documents the occurrence of antibodies to *N. caninum* in wild canids from PEI, however the observed seroprevalence in foxes (2.6%) and coyotes (2.5%) was low. The serologic evidence of *N. caninum* infection in the foxes and coyotes poses questions about the relative importance of transmission of *N. caninum* between wildlife and livestock.

Chapter 7 describes the findings of oocysts morphologically and morphometrically similar to oocysts of *N. caninum* in 2 fox and 2 coyote fecal samples. DNA was extracted from these samples and found positive on nested PCR utilizing primers to the *N. caninum*-specific Nc5 genomic sequence. PCR using primers specific for *Hammondia heydorni* failed to yield an amplification product from these DNA samples.

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List of Abbreviations

ANOVA	Analysis of variance
AVC	Atlantic Veterinary College
bp	base pair
CCC	Concordance correlation coefficient
cELISA	Competitive enzyme-linked immunosorbent assay
CI	Confidence interval
DAT	Direct agglutination test
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
GIS	Geographical information system
h	hour(s)
iELISA	Indirect enzyme-linked immunosorbent assay
IFAT	Indirect fluorescent antibody test
IgG	Immunoglobulin G
NAT	*N. caninum*-agglutination test
nt	nucleotide
OD	Optical density
PABAK	Prevalence adjusted bias adjusted kappa
PCR	Polymerase chain reaction
PEI	Prince Edward Island
s	second(s)
S/P ratio	Sample to positive ratio
SD	Standard deviation
Se	Sensitivity
Sp	Specificity
TG-ROC	Two-graph receiver operating characteristic

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1. GENERAL INTRODUCTION

1.1. *Neospora caninum*

Neospora caninum is an intracellular protozoan parasite of the phylum Apicomplexa and class Coccidia (Dubey et al., 2002). The parasite was first discovered in dogs with neurological disorders (Bjerkås et al., 1984; Dubey et al., 1988). Prior to its discovery, *N. caninum* was probably mistaken for the closely related parasite *Toxoplasma gondii.* A few years later *N. caninum* infection was also described in cattle (Anderson et al., 1991; Thilsted et al., 1989). During the 1990's, several studies have found that the parasite is an important cause of bovine abortion in many parts of the world and that both acutely and chronically infected cattle have an increased risk to abort. *N. caninum* can exist in two different forms within the cells of dogs and cattle: the actively proliferating, disease-causing, tachyzoite stage, and the bradyzoite stage that lies dormant in tissue cysts. A third form of the parasite, the oocyst stage, is a sexually produced and environmentally resistant form that is responsible for the transfer of the infection from animal to animal, and therefore this form exists external to the host. Description of the pathogenesis and clinical effects of *N. caninum* infection, as well as the parasite's structure and biology, and the host-parasite relationship, are reviewed in Dubey and Lindsay (1996) and Dubey (2003).

1.2. Lifecycle: definitive hosts, intermediate hosts

The structural similarities to *T. gondii* led researchers to suspect that *N. caninum* had a

similar life-cycle, with intermediate hosts and a carnivorous definitive host. Before the life-cycle was known, findings in epidemiological studies indicated that the presence of dogs on a farm increased the risk of *N. caninum* abortion in cattle (Wouda et al., 1999). In 1998, experimentally infected dogs were shown to excrete *N. caninum* oocysts with their feces (Dijkstra et al., 2001b; McAllister et al., 1998). In 2004, the coyote (*Canis latrans*) was found to be an additional definitive host (Gondim et al., 2004b). Exposure to coyotes or grey foxes (*Urocyon cinereoargenteus*) was also identified as a risk factor for *N. caninum* in a previous epidemiological study (Barling et al., 2000). It is assumed that there may be other definitive hosts in wildlife and some common species have been investigated (Dubey, 1999; Dubey, 2003). For example, foxes can carry the parasite (Schares et al., 2001), but it has not been verified that foxes shed oocysts (Schares et al., 2002), i.e. are actual definitive hosts. In Belgium and Spain antibodies to *N. caninum* were found in >17% and 11% of samples from foxes (Almeria et al., 2002; Buxton et al., 1997), while no seropositive samples were found when Swedish red foxes were surveyed (Jakubek et al., 2001). However, a seroprevalence ranging from 0.9 to 44% has been observed in foxes from the UK, Austria, Argentina, Israel and Spain (Almeria et al., 2002; Hamilton et al., 2005; Martino et al., 2004; Steinman et al., 2006; Wanha et al., 2005). Two studies on seroprevalence of *N. caninum* in coyotes were carried out in Illinois and Texas, US (Gondim et al., 2004a; Lindsay et al., 1996) where a seroprevalence of 10-11% was observed. No North-American studies on the seroprevalence for *N. caninum* in red foxes (*Vulpes vulpes*) have been reported.

Although the consequences of *N. caninum* infection presently seem to be of highest importance in cattle, several other species, such as brown rats, raccoons, deer, horses and

water buffalo, are also possible intermediate hosts of *N. caninum* and could potentially experience negative effects as a result of infection (Dubey, 2003). Other possible hosts of interest are, of course, humans. Sera from farmers and aborting women have been examined for presence of antibodies, but no clearly positive samples were found (Graham et al., 1999; Petersen et al., 1999). However, a recent study found antibodies to *N. caninum* in 38% of HIV-infected patients (Lobato et al., 2006). These findings might bring a new concern for the actual role of *N. caninum* infection in immunocompromised patients.

1.3. Transmission of the parasite in cattle

Cattle can be infected with *N. caninum* in two ways; 1) by transmission of the parasite from the cow to her fetus during gestation and 2) by transmission of the parasite via the feces from a definitive host. The optimal study design for investigating the degree of vertical transmission is to obtain pairs of precolostral samples of the newborn calf and its mother. These studies are rare, but have been performed, and show that vertical transmission occurs in 68-95% of gestations in *N. caninum* infected cattle (Davison et al., 1999; Paré et al., 1997; Wouda et al., 1998). Thus, the majority of calves born from infected cows are congenitally infected. There are also studies that have used more indirect measures of vertical transmission, e.g. by comparing the serological status of mother and offspring later in life. In these comparisons the probability of a seropositive offspring due to vertical transmission was 0-86% (Bergeron et al., 2000; Dijkstra et al., 2001a; Romero et al., 2003). The reason for the wide range of these estimates is that they depend not just on the degree of congenital infection, but also on the presence of

horizontal transmission and the time passed since the parasite was introduced into the herd.

In contrast to vertical transmission, the degree of horizontal transmission certainly differs between populations and over time. In this context, horizontal transmission refers to cattle being infected by ingestion of oocysts excreted by a definitive host. Other types of horizontal transmission have been investigated but none has been found to occur naturally. Newborn calves fed colostrum spiked with tachyzoites can be infected with *N. caninum* (Uggla et al., 1998). However, none of the calves that were fed colostrum from infected cows were infected, and consequently it was concluded that this route of transmission is not of major importance (Davison et al., 2001). Three dogs fed *N. caninum*-infected placenta did shed *N. caninum*-oocysts, but no oocyst shedding was observed in two dogs that were fed colostrum spiked with *N. caninum* tachyzoites (Dijkstra et al., 2001b). Interestingly, none of the dogs produced serum *N. caninum* antibodies. In addition, the parasite has never been detected in colostrum of infected cows. Other researchers have suggested that the ingestion of placenta or amniotic fluid expelled by infected cows could result in horizontal transmission between cattle (Bergeron et al., 2001; Davison et al., 2001), but this has so far not been verified.

1.4. Diagnosis of *N. caninum*

According to current knowledge, infected individuals remain carriers of *N. caninum* for life. It is also assumed that all infected cattle continue to produce specific antibodies. However, fluctuations of antibody levels have been observed in both experimentally and naturally infected cattle. In particular during gestation, the interaction between the hosts'

immune system and the parasite seems to result in rapid peaks and drops in detectable antibody levels. On occasion, infected cattle antibody levels can fall below the cut-off levels of the commonly used serological assays (Sager et al., 2001; Trees et al., 2002).

Several assays have been developed to detect *N. caninum* antibodies in serum. These serological tests include indirect fluorescent antibody tests (IFAT), enzyme-linked immunosorbent assays (ELISAs), immunoblotting and *N. caninum*-agglutination tests (NAT) (Björkman et al., 1999). While several commercial tests are available, many laboratories with a *N. caninum* research program, or that are involved with *N. caninum* diagnostics, have also developed their own immunoassays using their own cut-off values or other criteria for interpretation. Thus, the interpretation of data from different laboratories depends on the quality of the serological diagnostic tools and the utilized cut-off value, making it difficult to compare data from different laboratories using different (or even the same) techniques. Substantial variability in agreement amongst assays used on cattle has been described in the USA (Dubey et al., 1997) and Canada (Waldner et al., 2004). However, these studies only used a small set of reference sera or did not have information on the 'true' status of the sera. In contrast, a large study showed a high level of agreement in the interpretation of test results in cattle from 6 commercial and 6 in-house tests used in Europe (von Blumröder et al., 2004).

Antibody detection by serology is commonly used as a first method of screening wildlife for *N. caninum*. However, investigating wildlife is challenged by difficulties with sample collection. Samples are often derived from carcasses showing varying degrees of decomposition and the level of hemolysis of serum from decomposed animals might be variable. The question arises as to whether these sera are still appropriate for serological

testing, because the reliability and performance of immunoassays has been questioned (Lillehaug et al., 2003). Some sampling and storage factors might artificially induce hemolysis, such as prolonged storage of the blood before separation, rapidly forcing blood through small needles, excessive agitation when mixing, the physical act of centrifugation and separation of serum, or an improper storage temperature (O'Neill et al., 1989). Although several seroprevalence studies for *N. caninum* in wildlife have been published, scarce information is available on the quality of *N. caninum* antibodies in animal blood with different degrees of hemolysis. An experiment, using an indirect enzyme-linked immunosorbent assay (ELISA) with an *N. caninum* immunostimulating complex for cattle sera, showed that specific IgG antibodies were stable in sterile blood and decomposed lung tissue when stored at room temperature for up to 118 days (Jakubek et al., 2005). A reduced specificity was observed using severely hemolysed serum from 19 mountain lions in a *N. caninum* modified agglutination test (Packham et al., 1998). The significance of using non-hemolysed serum for antibody analysis, especially when dealing with wildlife, is important prior knowledge for adequate data collection.

Compared to individual serum samples, the collection of bulk milk samples (comprising a pooled sample of milk from all lactating cows in a herd) is a non-invasive, convenient and economical method of sampling and testing groups of dairy cattle. In Europe and Asia, studies have been performed to evaluate the test characteristics of enzyme-linked immunosorbent assays (ELISAs) for *N. caninum* on individual and bulk milk samples (Bartels et al., 2005; Björkman et al., 1997; Chanlun et al., 2002; Frössling et al., 2006; Schares et al., 2004; Schares et al., 2003). In previous work, an indirect

ELISA on individual milk samples demonstrated a 90% sensitivity and 90% specificity relative to serum (Schares et al., 2004). Bartels et al. (2005) evaluated the application of the same indirect ELISA on bulk milk samples and found a herd-level sensitivity and specificity of 61 and 92%, respectively. A recent study from Thailand showed that repeated bulk milk testing at regular intervals provided better information about herd *N. caninum* status than a single test (Chanlun et al., 2006). However, no studies have been reported in North America evaluating the test characteristics and the variation over time of *N. caninum* antibodies in bulk milk.

1.5. Epidemiology of *N. caninum* on Prince Edward Island

In a study performed between 1998 and 2003, the overall Canadian cow-level seroprevalence of *N. caninum* was 11.9% and herd-level seroprevalence was 81.9% (Haddad, 2006). Economic losses due to *N. caninum* in the Canadian dairy industry were estimated to be around $1500 per year for a 61-cow herd, but there was a wide range, mainly due to variation in the within-herd seroprevalence (Haddad, 2006). In a serological survey performed in 1998, 63% of the Prince Edward Island (PEI) dairy farms had at least two *N. caninum*-positive cows (Keefe and VanLeeuwen, 2000). Due to the potential economic impact of *N. caninum*, particularly on the breeding and selling of high quality dairy cattle on PEI, there is a great deal of interest in controlling *N. caninum* on dairy farms. Currently, there is no monitoring program for *N. caninum* on PEI and producers might not be aware of the impact *N. caninum* might have on their farms. Epidemic outbreaks of *N. caninum* will likely be identified as such, but endemic abortions might not be brought to the attention of, for example, a producer's herd health

veterinarian. Considering the horizontal and vertical routes of transmission for *N. caninum*, it is likely that disease prevalence might increase over time if no management measures are taken (French et al., 1999). A monitoring program where farms are tested on a regular basis in connection with already existing milk quality testing will be an economical way to identify farms that will benefit from intervention. The investment for the producer to monitor *Neospora*-status on the farm can therefore be minimal. The Dutch Animal Health Service successfully started a *Neospora*-monitoring program in 2004 that has been received well by producers (Dijkstra et al., 2005).

Prince Edward Island consists mainly of agricultural and forested land where coyotes and foxes co-occupy habitat throughout the island. It is estimated that the density of foxes and coyotes in the province is 1 and 0.4 animals/km^2, respectively (R. Dibblee, pers. comm.). Due to the relatively high density and close proximity of foxes, coyotes and cattle in the province, the two canid species are sighted close to farms and have access to carcasses and placentas. Therefore, these two wild canid populations on PEI are potential reservoirs of *N. caninum*, but this has never been investigated. More knowledge is required about these populations to confirm them as risk factors for diseases such as *N. caninum*, to include them within control strategies, and to verify if control strategies have an effect in the future.

1.6. Aim and scope of the thesis

The overall goal of this thesis project was to investigate the potential sylvatic life cycle of *N. caninum* and its epidemiology on PEI, Canada. This work required validation of diagnostic tests for dairy herds and wild canids. Validated tests could then be applied

to characterize herd status and to examine status of wild canids.

More specifically, the aims were:

- To validate and use an indirect enzyme-linked immunosorbent assay in bulk milk to estimate the prevalence of *Neospora caninum* on dairy farms on Prince Edward Island, Canada
- To study the population structure and habitat of the wild fox (*Vulpes vulpes*) and coyote (*Canis latrans*) on Prince Edward Island, Canada
- To evaluate serological methods used in North America for the diagnosis of *Neospora caninum* infection in cattle
- To determine the effect of hemolysis on the performance of two *Neospora caninum* enzyme-linked immunosorbent assays using cattle and fox sera
- To evaluate and use an enzyme-linked immunosorbent assay to determine the seroprevalence of *Neospora caninum* in foxes (*Vulpes vulpes*) and coyotes (*Canis latrans*) on Prince Edward Island, Canada
- To investigate the occurrence of *Neospora caninum* oocysts in feces of free-ranging red foxes (*Vulpes vulpes*) and coyotes (*Canis latrans*)

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2. USE OF AN INDIRECT ENZYME-LINKED IMMUNOSORBENT ASSAY IN BULK MILK TO ESTIMATE THE PREVALENCE OF *NEOSPORA CANINUM* ON DAIRY FARMS ON PRINCE EDWARD ISLAND, CANADA

2.1. Introduction

Neospora caninum is an important cause of sporadic, epidemic and endemic abortion in cattle worldwide (Dubey et al., 1996). The infection usually has a chronic course and persists throughout the life of an infected animal (Björkman et al., 1996). Thus far, no vaccine is available that limits endogenous transplacental infection (Trees et al., 2005), and there is no treatment that prevents or cures *N. caninum* infection. Consequently, the strategy to reduce the prevalence and the losses caused to the farming industry by *N. caninum* is to break the life cycle of the parasite and eliminate infected animals (Frössling et al., 2005; Wouda et al., 1998).

Diagnosis of neosporosis in dairy cattle is achieved by detection of *N. caninum* antibodies in serum or milk. Previous research has suggested that farms with a within-herd seroprevalence ≥15% have an increased risk for reproductive losses (Dijkstra et al., 2001; Wouda et al., 1999). Therefore, a 15% within-herd seroprevalence is considered an appropriate cut-off value for identifying a herd with substantial reproductive losses due to neosporosis (Bartels et al., 2005).

Compared to individual serum samples, the collection of bulk milk samples (comprising a pooled sample of milk from all lactating cows in a herd) is a non-invasive, convenient and economical method of sampling. Diagnostic tests adapted for use with bulk milk have been developed for several viral (Elvander et al., 1995; Forschner et al., 1989; Paton et al., 1998; Pritchard et al., 2002; Traven et al., 1999), bacterial (Dom et al.,

1991; Grove et al., 1992; Nielsen et al., 2005; Nielsen et al., 2000; Paiba et al., 1999; Thoen et al., 1995; Veling et al., 2001) and parasitic (Charlier et al., 2005; Kloosterman et al., 1996; Sanchez and Dohoo, 2002) bovine diseases. Today, bulk milk analysis is routinely used as a tool in diagnosis of bovine herpes virus and bovine viral diarrhea virus infections in dairy herds in Scandinavia (Lindberg et al., 1999; Niskanen et al., 1991; Nylin et al., 1999).

In Europe and Asia, studies have been performed to evaluate test characteristics of enzyme-linked immunosorbent assays (ELISAs) for *N. caninum* on individual and bulk milk samples (Bartels et al., 2005; Björkman et al., 1997; Chanlun et al., 2002; Frössling et al., 2006; Schares et al., 2003; Schares et al., 2004). In previous work, an indirect ELISA on individual milk samples demonstrated a sensitivity and specificity relative to serum of 90%, and a linear correlation between milk and serum antibody results was characterized by an $R^2 = 0.70$ (Schares et al., 2004). Bartels et al. (2005) evaluated the application of the same indirect ELISA on bulk milk samples and found a sensitivity and specificity of 61 and 92%, respectively. A recent study from Thailand showed that repeated bulk milk testing at regular intervals provided better information about herd *N. caninum* status than a single test (Chanlun et al., 2006). However, no studies have been reported in North America evaluating test characteristics and the variation over time of *N. caninum* antibodies in bulk milk.

In a serological survey performed in 1998, 63% of the Prince Edward Island (PEI) dairy farms had at least two *N. caninum*-positive cows (Keefe and VanLeeuwen., 2000). Due to the potential economic impact of *N. caninum* (Trees et al., 1999), particularly on the breeding and selling of high quality dairy cattle on PEI, there is a great deal of interest

in screening dairy farms for *N. caninum*. Thus, diagnostic tests utilizing bulk milk rather than individual serum would be valuable, cost-effective tools for identifying farms that may benefit from further investigation and implementation of control measures.

The objectives of this study were: 1) to evaluate the use of bulk milk as a diagnostic tool for estimation of herd-level *N. caninum* exposure, 2) to estimate the prevalence of dairy farms on PEI with a ≥15% within-herd *N. caninum* seroprevalence using bulk milk, and 3) to study the variation over time of *N. caninum* antibodies in bulk milk.

2.2. Material and methods

2.2.1. Sample population and collection of samples and data

Bulk milk samples from PEI dairy farms were collected in May 2004, May 2005, and June 2005. In May 2004 and June 2005, 20-ml bulk milk samples from 235 farms (all but 2 of the dairies in PEI in June 2005) were obtained for laboratory testing. In May 2005, because of technical difficulties, bulk milk samples were obtained from only 189 farms. No information on abortion history was available from these herds.

The bulk milk samples were centrifuged at 1000 X *g* for 10 min. Skimmed milk from beneath the cream layer was obtained from the milk samples and stored at -20ºC until analyzed. At analysis, the thawed skimmed milk aliquots of bulk milk samples were assayed for the presence of antibodies to *N. caninum,* using a commercially available indirect ELISA (Herd Check Anti-*Neospora* ELISA, IDEXX Laboratories, Westbrook, Maine, USA) and the manufacturer's recommendations, with 1 exception; as described by Bartels et al.(2005), milk samples were diluted 1:2 instead of 1:100 as recommended for serum.

The positive and negative control sera provided with the test kit were used as controls at the 1:100 dilution recommended by the manufacturer. Test results were expressed as a sample-to-positive (S/P) ratio. The S/P ratio was defined as the optical density (OD) of the sample (S) minus the OD of the negative control (NC), all divided by the OD of the positive control (PC) minus the OD of the negative control ((S-NC)/(PC-NC)). Milk samples with a S/P ratio ≥0.6 were considered positive, thus indicating a farm with an estimated within-herd seroprevalence of 15% or more, suggesting that these farms have in increased risk for reproductive losses, as reported by Bartels et al. (2005).

To evaluate the use of the ELISA on bulk milk as a diagnostic tool for estimation of herd-level *N. caninum* exposure in Atlantic Canada, an in-house (AVC) validation process of this ELISA under our laboratory conditions was conducted. The validation utilized 31 reference bulk milk samples that were provided by the Animal Health Service (AHS) in Deventer, The Netherlands.

For 11 farms that were considered positive for *N. caninum* on one or more bulk milk samples, 10-ml blood samples were collected from all adult cows (ranging from 34 to 104 per farm) in September 2005. At this visit, a 4th bulk milk sample was also collected. During the visit, the stage of lactation and whether each cow's milk was included in the bulk tank was recorded.

The blood samples were centrifuged at 1000 X *g* for 10 min. Serum was harvested from the blood samples, and stored at -20ºC. Serum samples were analyzed for *N. caninum* antibodies by using a commercially available competitive-inhibition ELISA (VMRD, Pullman, Washington, USA) according to the manufacturer's recommendations. Sera with an inhibition value > 30% were considered positive. The percentage inhibition

was calculated as 100-[(OD of test sera/mean OD of negative reference sera) X 100]. The reported sensitivity and specificity of this test (using serum) are 97.6% and 98.6%, respectively (Baszler et al., 2001). Samples from the 4th bulk milk sampling were handled and tested in a similar manner to the first 3 bulk milk samples.

Serum and milk samples were stored at -20ºC and analyzed on 10 separate days during the study period when batches of at least 45 samples would be available for analysis. Serum and milk samples were analyzed in duplicate and the OD was measured in a microplate spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA, USA) at a wavelength of 650 nm.

2.2.2. Statistical analyses

A concordance correlation coefficient (CCC) (Lin, 1989) was calculated to estimate the level of agreement between reference bulk milk samples performed in the two laboratories, and between the bulk milk ELISA results among the sampling dates. A Pearson's correlation coefficient was calculated to determine the correlation between within-herd seroprevalence and bulk milk ELISA results of the 11 farms that were test-positive on bulk tank milk and were individually tested for cows positive for *N. caninum*. A one-way repeated measures analysis of variance (ANOVA) was used to determine a difference between the mean S/P ratio on the three sampling dates. Scatterplots were created to assist in the visual representation of the data. Stata version 8 (Stata Corporation, College Station, TX, USA) was used for the statistical analyses of the data.

2.3. Results

2.3.1. Reference bulk milk samples

ELISA results on 31 reference samples correlated well between the two laboratories; the CCC was 0.82 (95% CI: 0.73-0.91) (Fig. 2.1). Only 3 samples were classified as negative at the Animal Health Service in The Netherlands but classified positive at the Atlantic Veterinary College on PEI. All three samples had ELISA results very close to the cut-off value (0.60) for one of the two tests (0.70, 0.68 and 0.59), making their interpretation challenging.

2.3.2. Bulk milk from dairy farms on PEI

In May 2004, 15 of 235 bulk milk samples (6.4%, 95% CI: 3.2-9.5%) were ELISA-positive, while in May 2005 and June 2005, respectively, 19 of 189 (10.1%, 95% CI: 6.2-15.3%) and 24 of 235 (10.2%, 95% CI: 6.3-14.1%) farms were found to be positive (Fig. 2.2 and 2.3). A one-way repeated measures ANOVA revealed a slight trend in increase in S/P ratio over time (p=0.11). The CCC was 0.25 (95% CI: 0.13-0.37) on samples collected in May 2004 and June 2005. The CCC increased to 0.36 (95% CI: 0.23-0.48) and 0.73 (95% CI: 0.67-0.80), comparing samples collected in May 2004 and May 2005, and in May 2005 and June 2005, respectively. From 15 ELISA-positive farms in May 2004, 5 farms remained positive in both May and June 2005. Seven farms that were positive in 2004 were negative on both samplings in 2005.

Regarding variation in test results from the bulk milk, 161 farms of the 189 farms that had results for May 2004 and May and June 2005 showed consistent test results, with 5 (3%) farms being positive for all 3 sampling occasions and 156 (83%) farms being negative on all 3 occasions.

2.3.3. Individual serology and bulk milk from 11 selected farms

Results for the 11 selected farms, which were ELISA-positive on bulk milk for one or more occasions in May 2004, May 2005 and June 2005 are shown in Table 2.1. The Pearson's correlation coefficient between serology and bulk milk samples collected in September 2005 was 0.87 (95% CI: 0.57-0.97) (Fig. 2.4). When using the interpretation of bulk milk ELISA results with respect to seroprevalence levels, proposed by Bartels et al. (2005), 10 herds of the 11 herds were classified correctly when using the established cut-off value of 0.60 (Table 2.1). Only Farm 6 did not classify correctly; Farm 6 was negative according to the fourth bulk milk sample (S/P ratio=0.5) but had a within-herd seroprevalence of slightly more than 15%, at 15.4% (Table 2.1).

2.4. Discussion

The results of this study confirm that the prevalence of *N. caninum* in dairy farms can be estimated with the bulk milk ELISA used in this study. A previous evaluation of this test determined a high specificity (92%) but a limited sensitivity (61%), when applying a cut-off value of 0.6 (Bartels et al., 2005). Because of the moderate sensitivity, a considerable part of dairy herds tested will be incorrectly classified as negative. Dependent on the purpose of a screening test, this can be an important disadvantage. The test sensitivity can be increased by repeated bulk milk testing (Chanlun et al., 2006). The frequency of repeated testing to increase the sensitivity will depend on dynamics of the infection and dynamics in the lactating herd. The association between seroprevalence level and risk for reproductive losses may be different in different dairy industry

situations. The herd-prevalence of *N. caninum* in The Netherlands is 76% in dairy herds (Bartels et al., 2006), comparable to 79% in dairy farms in Atlantic Canada (Keefe and VanLeeuwen, 2000). As the dairy industry in The Netherlands and Atlantic Canada are comparable, this cut-off value was considered appropriate in this study. The CCC of 0.82 between the two laboratories suggests that extrapolation of the S/P ratio cut-off value of 0.6, as proposed by Bartels et al. (2005) is adequate for an Atlantic Canadian context. Differences in S/P ratios may be caused by different laboratory techniques when performing an ELISA, such as automated versus manual washing steps. Using more reference samples to optimize a cut-off value for each laboratory situation would be ideal, but may not be feasible in a practical setting.

Investigating 11 farms using serology in combination with bulk milk further confirmed that the cut-off value of 0.6 corresponded well with a within-herd seroprevalence of 15% (correlation coefficient=0.87). Our main concern was in correctly classifying bulk milk ELISA-positive farms, and for this reason, bulk milk ELISA-positive farms were selected for individual serology. Because of economic reasons (in-kind contribution of competitive ELISA-kits), a competitive ELISA was used to test serum, while an indirect ELISA was used for the bulk milk analysis.

There was individual farm variability in S/P ratios in bulk milk over time. The difference over time in bulk milk ELISA results is likely the result of test-positive cows leaving or entering the milking herd. In addition, an active infection in the herd, such as a point source infection, may also lead to a sudden increase in S/P ratio caused by an increased antibody response. The bulk milk samples only represent cows that contributed milk to the bulk tank on the day of sampling, and do not include dry cows, sick cows or

cows in the colostral period. If these cows, or the young stock of the herd, were the majority of seropositive animals, the bulk milk test may not detect the herd as positive. However, performing multiple tests per year would compensate for this scenario. Another reason for dissimilarity between prevalence of antibody positive cows and total antibodies in bulk milk could be antibody levels, lactation stage, and milk yield per animal. This is likely to be more important in small herds with few cows attributing to the bulk milk (Chanlun et al., 2002). Lactation stage was identified as a factor that was associated with an increase in the milk *N. caninum* ELISA result relative to serum in individual animals (Schares et al., 2004). We know from previous studies that *Neospora*-antibody titres in serum can fluctuate over time (Jenkins et al., 1997), and it is therefore likely that antibody titres in milk will fluctuate as well. Despite these factors, there was good correlation between seroprevalence and the bulk milk ELISA S/P ratio performed on samples collected at the same day.

The proportion of bulk milk ELISA-positive farms on PEI tended to increase between May 2004 and June 2005. However, this increase was borderline significant and other factors besides increased seroprevalence may have played a role. Variation between ELISA-plates and variation between test days has to be considered, as well as other farm level factors. The CCC increased considerably when comparing sampling rounds that were closer in time (0.36 and 0.25 when 12 and 13 months apart, versus 0.73 when one month apart). Bulk milk sampled after a one-month interval is likely to be more similar compared to a bulk milk sample collected after a 1-year interval, due to dynamics in a dairy herd. A more detailed study (including factors at the farm and laboratory level) monitoring *N. caninum* antibody levels in milk and serum over time could provide more

insight in the variability observed in this study.

A monitoring program where farms are tested on a regular basis in connection with already existing milk quality testing will be an economical way to identify farms that will benefit from intervention. The investment for the producer to monitor *Neospora*-status on the farm can therefore be minimal. The Dutch Animal Health Service successfully started a *Neospora*-monitoring program in 2004 which has been received well by producers (Dijkstra et al., 2005). In conclusion, this bulk milk ELISA can be used to identify dairy farms with a 15% within-herd seroprevalence in Atlantic Canada. Repeated sampling is recommended and more research is necessary to possibly control factors involved in the observed variability.

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Table 2.1. Comparison of *Neospora caninum* ELISA results from serum (cow level, 1sampling date) and bulk milk (herd level, 4 sampling dates) from 11 dairy farms.

	Bulk Milk ELISA S/P Ratio [a]				Cow Level Serum ELISA Test Results [b]		
Herd	May '04	May '05	June '05	Sept '05	Prevalence (%)	# positive cows	total # cows
1	0.04	0.25	**0.64**	0.15	1.3	1	77
2	0.34	**0.82**	**0.62**	0.25	5.7	2	35
3	**0.85**	0.28	0.31	0.40	1.5	1	66
4	**0.61**	0.28	0.33	0.41	5.6	4	72
5	0.50	0.47	**0.64**	0.41	9.8	4	41
6	**0.65**	0.21	0.14	0.50	15.4	4	26
7	0.43	**0.70**	**0.65**	**0.67**	17.5	7	40
8	**0.67**	0.54	0.34	**0.78**	17.9	7	39
9	0.42	**0.64**	**0.64**	**0.99**	27.4	23	84
10	0.55	**0.69**	**0.60**	**1.00**	30.6	22	72
11	0.14	**1.01**	**0.75**	**1.09**	17.9	12	67

[a] Sample-to-Positive Ratio - Bold printed numbers indicate a positive bulk milk result (S/P $\geq$ 0.60).

[b] Individual serum samples were collected on the same day as the bulk milk sample in September 2005. All numbers refer only to lactating cows contributing to the bulk tank on the sampling date.

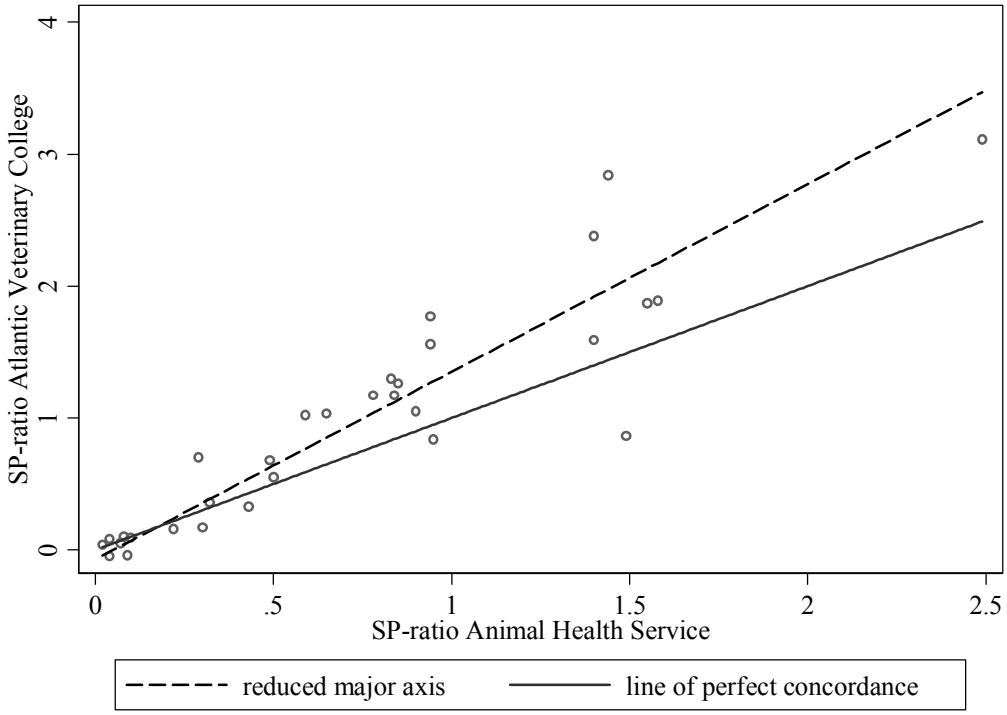

Figure 2.1. Scatterplot of results from a *Neospora caninum* indirect ELISA performed on 31 reference bulk milk samples analysed at the Atlantic Veterinary College, Canada and at the Animal Health Service, The Netherlands. Solid line represents perfect agreement whereas dashed line indicates the line of best fit for the relationship between the test results.

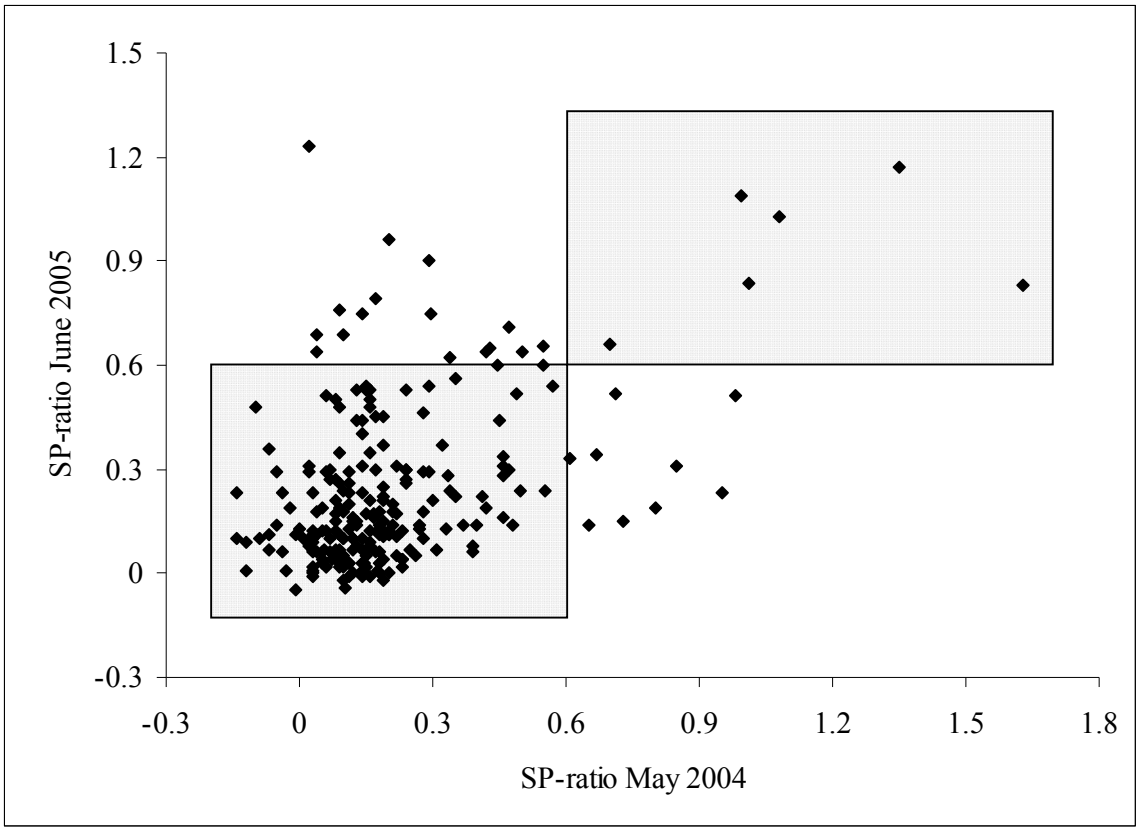

Figure 2.2. Scatterplot of results from a *Neospora caninum* indirect ELISA performed on two bulk milk samples collected more than one year apart (May 2004 and June 2005) from 235 dairy farms on Prince Edward Island. The shaded areas contain farms that had the same test result (positive or negative) on both occasions, based on a cut-off value of ≥0.60.

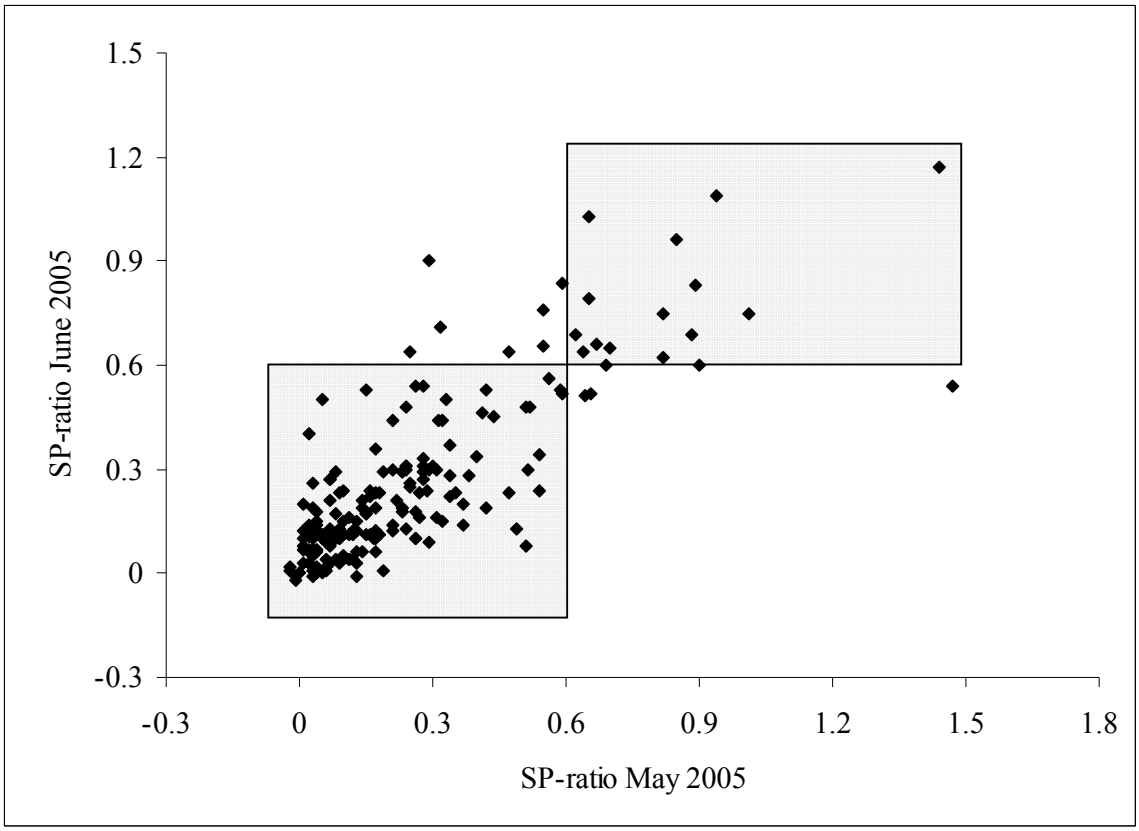

Figure 2.3. Scatterplot of results from a *Neospora caninum* indirect ELISA performed on two bulk milk samples collected one month apart (May and June 2005) from 189 dairy farms on Prince Edward Island. The shaded areas contain farms that had the same test result (positive or negative) on both occasions, based on a cut-off value of $\geq$0.60.

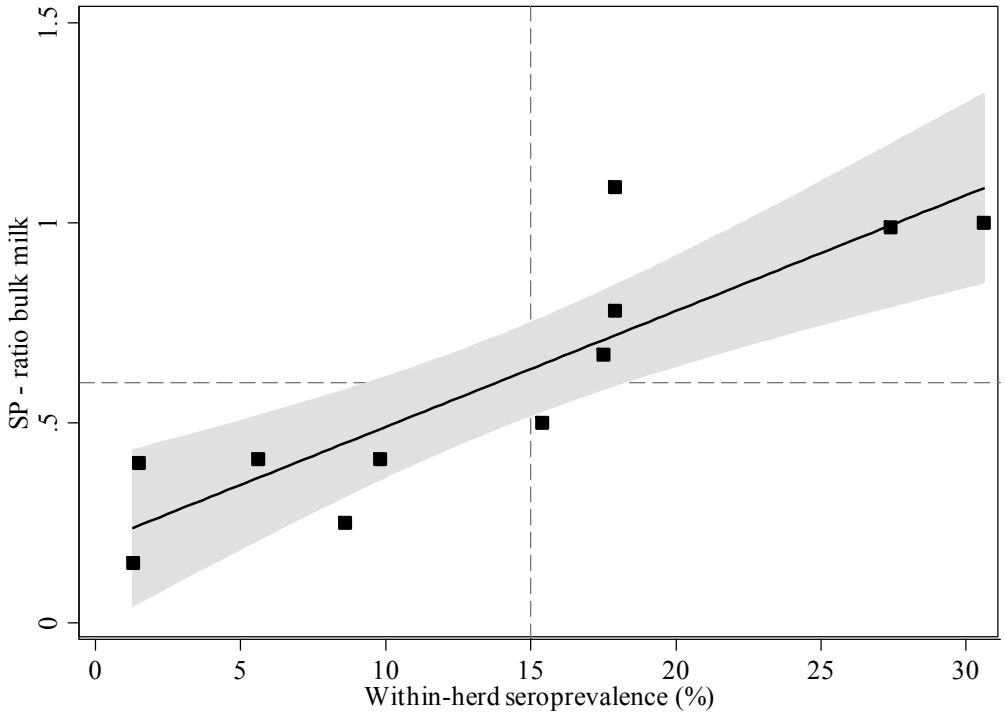

Figure 2.4. Scatterplot of bulk milk results from a *Neospora caninum* indirect ELISA versus within-herd seroprevalence results from a *Neospora caninum* competitive ELISA of the lactating cows that were contributing to the bulk tank, from 11 dairy farms on Prince Edward Island. Bulk milk and blood sample collection were performed on the same day in September 2005. Dashed lines represent a cut-off value of 0.60 for the S/P ratio and 15% for within-herd seroprevalence. The shaded area represents a 95% confidence interval around the best line of fit.

3. COMPARISON OF SEROLOGICAL METHODS FOR THE DIAGNOSIS OF *NEOSPORA CANINUM* INFECTION IN CATTLE

3.1. Introduction

Definitive diagnosis of *Neospora*-associated abortions is based on examination of the aborted foetus, including observation of characteristic lesions, combined with immunoperoxidase staining or PCR in foetal tissues (Wouda et al., 1997). However, in many instances, foetal material is not available. In these situations, a presumptive diagnosis can be achieved based on *N. caninum* antibody detection by serological assays.

Serological techniques available to detect specific antibodies to *N. caninum* infection include indirect fluorescent antibody tests (IFATs), a variety of enzyme linked immunosorbent assays (ELISAs), and a *N. caninum*-agglutination test (NAT). In addition to the use of these assays for abortion-related diagnosis, the different serological techniques are also widely used in epidemiological studies and herd-level control efforts regarding *N. caninum*.

While several commercial tests are available, many laboratories with a *N. caninum* research program, or that are involved with *N. caninum* diagnostics, often develop their own immuno-assays, using their own cut-off values or other criteria for interpretation. Thus, the interpretation of data from different laboratories depends on the quality of serological diagnostic tools and the utilized cut-off value, making it difficult to compare data from different laboratories using different (or even the same) techniques. In 1997,

substantial variability between test results was observed in an evaluation of 5 ELISAs and one IFAT used in the US (Dubey et al., 1997). However, the sample size used to evaluate these tests was small (33 *N. caninum*-positive and 37 *N. caninum*-negative sera). In contrast, a large study recently showed a high level of agreement in the interpretation of test results from 6 commercial and 6 in-house tests used in Europe (von Blumröder et al., 2004). A Canadian study using field samples from beef cattle found good agreement between two ELISAs (κ=0.76) but much lower agreement (κ=0.46) between the same two ELISAs and an agglutination test (Waldner et al., 2004). However, in this study there was no information about the 'true' status of the sera.

The aims of this study were 1) to evaluate the performance and agreement of various commercial and in-house *N. caninum* antibody assays used in dairy cattle in North America, and 2) to investigate reproducibility of two assays performed in different laboratories.

3.2. Materials and Methods

3.2.1. Sample population

Serum samples were randomly collected from lactating dairy cows in three Canadian provinces (New Brunswick, Nova Scotia, and Prince Edward Island) as part of a large study in Canada in 1998 (Keefe & VanLeeuwen, 2000). No abortion history of these animals was known. From a sample pool of 2594 samples from 90 herds, 183 seronegative and 214 seropositive samples, as determined by a single serological assay ELISA-B1 (*N. caninum* indirect ELISA, Biovet Inc., St. Hyacinthe, Quebec, Canada), were randomly selected. The 397 sera in the final sample population originated from 34

herds. After collection in 1998, samples were stored in a -20ºC freezer in sealed containers to prevent dehydration.

3.2.2. Laboratory analyses

In total, 3 ELISAs, 2 IFATs and 1 NAT were utilized to test the sample population for antibodies to *N. caninum* (Table 4.1). The assays were performed according to the manufacturers' instructions or, in the case of in-house tests, according to respective laboratory protocols. ELISA-B2 had two recommended cut-off values, giving results classified as 'suspicious' if the sample-to-positive (S/P) ratio was between these two cut-off values.

In order to determine the reproducibility of two assays in different laboratories, the competitive ELISA (ELISA-A1and ELISA-A2) and the indirect ELISA (ELISA-B1 and ELISA-B2) were performed twice. The first competitive ELISA (ELISA-A1) was performed at the Atlantic Veterinary College (AVC, Prince Edward Island, Canada) in 2003, and ELISA-A2 by the VMRD laboratory (Pullman, WA, USA) in 2005. The first indirect ELISA (ELISA-B1) was performed in 1998 by the Biovet laboratory (Quebec, Canada), and ELISA-B2 by AVC in 2005. These two assays were selected for this reproducibility assessment because they are two commonly used commercial ELISAs for *N. caninum* in North America.

3.2.3. Gold standard determination

Determination of test operating characteristics of the assays under evaluation in this study required a comparison of the test results with the 'true state' of the sample

population. In order to determine this 'true state' in the current study, one of the test assays was validated on 452 reference sera provided by the *Neospora*-research group in Europe (von Blumröder et al., 2004). As the volume of sera from this reference panel was limited, only one test, the IFAT-A (Table 4.1), could be performed with these reference sera. If the IFAT-A resulted in a high sensitivity and specificity using these reference sera, then the results from IFAT-A on the sample population could be used as reference standard for the sample population. These European reference sera were considered to be a 'reference panel' because the sera underwent a comparison of 12 European serological methods, reported by von Blumröder et al. (2004). A 'true state' of nature of the 452 sera (286 *N. caninum*-negative and 166 *N. caninum*-positive) was determined by 'majority of test-outcome', based on the decision (positive or negative) of the majority of the 12 assays. From these 452 samples, 234 samples (142 *N. caninum*-negative and 92 *N. caninum*-positive) also had 'pretest' information on the 'true state' of nature of the sample, based on historical information from the animal (e.g. the animal was known to have aborted a foetus infected with *N. caninum*). Therefore, the results of the IFAT-A could be validated with the 'true state' of the whole panel of reference sera, and the 234 samples with additional information.

For interpreting the results of the IFAT-A on the 452 reference sera (and 397 sample sera), a 1:200 serum dilution was used, and the intensity of fluorescent antibody bound to the periphery of the tachyzoite was recorded on a categorical scale from negative to strong fluorescence (0 to 4+) by an experienced reader (Dubey et al., 1996). Fluorescence of the periphery of the tachyzoite recorded as 0 or 1+ was considered as *N. caninum*-negative result. Fluorescence of the periphery of the tachyzoite recorded as >1+ was

considered *N. caninum*-positive. A 'strong positive' response was a strong fluorescence of the periphery of the tachyzoite (> 3+), and a moderate fluorescence of the periphery of the tachyzoite (≤ 3+) was interpreted as a 'weak positive' response. Based on results of IFAT-A on these reference sera, the IFAT-A was subsequently considered as reference standard for assessing the operating characteristics of the 5 other assays on the sample population used in our study.

3.2.4. Statistical analyses

Because the more frequently used Cohen's kappa becomes unstable when prevalence of disease or infection is very low (<0.2) or high (>0.8) (Dohoo et al., 2003), prevalence-adjusted bias-adjusted kappa (PABAK) (Byrt et al., 1993) was used to assess test agreement between 8 assay results (6 assays, with 2 assays conducted at 2 laboratories). Positive and negative agreements were calculated to assess in more detail where disagreements among test results occurred (Cicchetti et al., 1990). Based on the positive-negative classification of the reference standard (IFAT-A) for the sample population, sensitivity (Se), specificity (Sp) and Youden's index (Youden, 1950) of the other 5 assays were calculated. For the ELISA results, two-graph receiver operating characteristic (TG-ROC) plots were used to assess each assay's ability to discriminate the sample population in relation to its cut-off value (Greiner et al., 2000).

To assess reproducibility between ELISA-A1 and ELISA-A2, and between ELISA-B1 and ELISA-B2, Lin's concordance correlation coefficient (CCC) (Lin, 1989) was calculated and a concordance correlation plot was utilized to examine absolute agreement. Stata version 8 (Stata Corporation, College Station, TX, USA) was used for the statistical

analyses of the data.

3.3. Results

3.3.1. Test performance of IFAT-A relative to European reference panel

For reference sera with 'pretest' information (n=234), Se and Sp of the IFAT-A were 0.99 (95% CI: 0.97-1.00) and 0.97 (95% CI: 0.95-1.00), respectively. When using the 'majority of test-outcome' information (n=452), the Se and Sp were 0.97 (95% CI: 0.94-1.00) and 0.97 (95% CI: 0.95-0.99), respectively. The IFAT-A response was more frequently 'strong positive' on samples of experimentally infected cows (88%), compared to cows that experienced a natural infection (32%)($P<0.01$).

3.3.2. Test performance of 5 assays relative to IFAT-A as reference standard

Only 11% (n=44) of the 397 sera in the sample population were found to be *N. caninum*-positive with IFAT-A. Using IFAT-A results as reference standard for the sample population, Se and Sp ranged from 0.66 to 1.00 and 0.52 to 0.99, respectively (Table 3.2). The highest Youden's index was 0.89 for both ELISA-B2 and IFAT-B.

In Figure 4.1, TG-ROC graphs illustrate the change in Se and Sp in relation to the S/P ratio (ELISA-B1, ELISA-B2 and ELISA-C) or inhibition percentage (ELISA-A1and ELISA-A2). A cut-off value that realizes equal test parameters (Se=Sp) can be obtained at the intersection point of the two curves in each graph. The cut-off value recommended by the manufacturer of ELISA-C was close to the intersection point, at a Se and Sp of 0.93 and 0.94, respectively (Fig. 3.1).

The PABAK indicated the least agreement (0.06) between ELISA-B1 and NAT, and

maximum agreement (0.97-0.98) was observed amongst ELISA-A1, ELISA-A2 and ELISA-B2 (Table 3.3). Negative agreement was 'substantial' to 'high' between all tests (0.66-0.99), according to Cicchetti et al. (1990). Positive agreement was ranging from 0.25 (between ELISA-B1 and NAT) to 0.96 (between ELISA-A1 and ELISA-A2).

3.3.3. Reproducibility

Almost perfect concordance was observed between ELISA-A1 and ELISA-A2 (Fig. 3.2), with the line of best fit only slightly deviating from the line of perfect concordance, which is in agreement with the high CCC (0.90, 95% CI 0.89-0.92). The line of perfect concordance between ELISA-B1 and ELISA-B2 (Fig. 3.3) was dissimilar to the best line of fit, which is in agreement with the observed CCC (0.36, 95% CI 0.30-0.41).

3.4. Discussion

The results of this study show good performance characteristics of IFAT-A compared to the European reference panel and demonstrate agreement between several serological assays used in North America. The decision as to which assays to include in this study was determined by their relative frequency of use in research and diagnostic laboratories in North America. The number of samples used was sufficient to give a good estimate of test performance, although a higher number of positive samples would have improved the accurateness of sensitivity results.

IFAT-A had a high Se and Sp in both 'pretest' and 'majority' reference samples, confirming that it was possible to use IFAT-A as reference standard to estimate Se and Sp for the other assays. The experimentally infected animals had high IFAT responses,

while the naturally infected animals had a significant lower IFAT response, which is in agreement with a study by Matsushita et al. (1987). This emphasizes the importance of validating assays with field samples, as using only experimental samples for validation may overestimate test performance.

Test performance of most assays was good, except for ELISA-B1 and NAT. The Se and Sp of a particular test can be manipulated by choosing different cut-off values. However, our main goal was to assess test performance and agreement of assays frequently used in research and diagnostic laboratories according to recommended cut-off values, not optimization of a commercially sold test. Appropriateness of a cut-off value depends on the purpose of performing the test, and must take into account the consequences of false-positive and false-negative outcomes. A previous study, that also compared ELISA-B2 and ELISA-C (among others), found a higher kappa, Se and Sp compared to a competitive ELISA and immunoblotting analysis (Wu et al., 2002). However, a small reference sample set was used (n=30), making estimates of test performance less precise. In addition, optimal cut-off values were determined for each test based on these reference sera, and were different from the cut-off value recommended by the manufacturers. Most variability in test performance was observed with respect to Se. In other studies (Dubey et al., 1997; Lally et al., 1996; Wouda et al., 1998), discrepancies in Se among tests were revealed especially when low titer sera were tested, highlighting the importance of selecting appropriate cut-off values for each test and each application. Concentration and type of antibodies present in the serum of chronically infected animals may differ compared to acute infections (Björkman et al., 1999), and therefore may be detected differently by certain antigens. With a serum

dilution cut-off of 1:25 a moderate Se (0.66) for NAT was observed. In a recent study (Canada et al., 2004) an optimal cut-off value of 1:40 was determined for NAT. By using a lower dilution as a cut-off value, a higher Se and lower Sp was expected, but this was not observed (Table 3.2). The Youden's index was highest for ELISA-B2 and IFAT-B. This indicates that an IFAT or ELISA, when only taking test performance into account, unconstrained by factors such as cost and time, would be the preferred techniques to use. However, the subjective evaluation of fluorescence in the IFAT-technique has to be considered. Experience of the person determining the degree of fluorescence in the sample is critical, and reader fatigue does influence the outcome. Furthermore, IFATs are more time-consuming than ELISAs when analyzing large numbers of samples.

The NAT and the competitive ELISA have the advantage of not being host-species specific. As wildlife appears to be involved in the *N. caninum* epidemiology, and considering there may be intermediate or definitive hosts not yet discovered, the option to test a variety of species with the same test can be advantageous. Assuming similar diagnostic performance of these two tests in other species, our results indicate that because of a higher Se, the competitive ELISA would be the preferred test to use.

Reproducibility of ELISA-A1 with ELISA-A2 was excellent (CCC=0.9). The CCC of ELISA-B1 and ELISA-B2 was marginal (0.36), but this could be explained by an improvement of the laboratory protocol of this assay between the first and second analysis. A technical problem in the protocol of ELISA-B1 had been identified and corrected between the analysis of ELISA-B1 and ELISA-B2 (Dr. E. Cornaglia, pers. comm.). It is questionable whether the comparison of ELISA-B1 and ELISA-B2 is an appropriate reproducibility test, because improvements had been made to the test. This

was not known at the time of analysis, and the assay was commercially available under the same label and conditions in 1998 and 2004, and was therefore considered as a reproducibility test.

ELISA-B1 had a low Sp (0.52), combined with a Se of 1.00, thereby considerably overestimating the prevalence when used in an epidemiological study. In a previous field-study using ELISA-B1, the Se and Sp, based on the manufacturer's estimates, was 0.99 and 0.98 respectively (VanLeeuwen et al., 2002). An explanation for the high numbers of *N. caninum*-positive sera in the first assay performed in 1998 compared to other assays could be that antibody levels have diminished over the 6-year period of the study. However, a 2-year interval did not have any influence on sample analysis as shown by the high agreement between the first and second analysis of ELISA-A. Previous studies have shown that freezing does not have an appreciable influence on antibody levels (Wang et al., 1997), although frequent freeze-thaw cycles may decrease antibody titers in serum (Brey et al., 1994). Thus, it is likely that the 1998 estimated Se and Sp were inaccurate, and chances of misclassifying an uninfected animal as infected were considerable.

The performance characteristics observed for most assays in this study make them useful for screening antibodies to *N. caninum*.

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Table 3.1. Summary information and identification of *Neospora caninum* antibody assays used in North America that were evaluated in the study.

Test_ID	Trademark[a]	Method	Diagnostic laboratory[a]	Year of analysis	Antigen
Commercial					
IFAT-A	VMRD Inc. (Baszler et al., 1996)	IFAT	VMRD	2005	whole tachyzoites
ELISA-A1	VMRD Inc. (Baszler et al., 2001)	competitive ELISA (VMRD, 2006)	AVC	2003	surface protein antigen captured using a monoclonal antibody
ELISA-A2			VMRD	2005	
ELISA-B1	Biovet Inc. (Paré et al., 1995)	indirect ELISA (Biovet, 2006)	Biovet	1998	sonicated lysate of tachyzoites
ELISA-B2			AVC	2004	
ELISA-C	HerdChek IDEXX (Paré et al., 1995)	indirect ELISA	AVC	2004	sonicated lysate of tachyzoites
In house					
IFAT-B	(Dubey et al., 1988)	IFAT	USDA	2004	whole tachyzoites
NAT	(Romand et al., 1998)	NAT	USDA	2004	whole tachyzoites

[a]VMRD Inc., Pullman, WA, USA; Biovet Inc., St. Hyacinthe, Quebec, Canada; IDEXX Corp., Westbrook, ME, USA; USDA, Beltsville, MD, USA; AVC, Atlantic Veterinary College, Charlottetown, Prince Edward Island, Canada

Table 3.2. Test characteristics and Youden's index (J) of five *Neospora caninum* antibody assays using recommended cut-off values on sera from 397 dairy cows.

Test [a]	Cut-off value	Sensitivity (95% CI)	Specificity (95% CI)	J (95% CI)
ELISA-A1	≥30%	0.89 (0.79-0.98)	0.98 (0.97-1.00)	0.87 (0.77-0.96)
ELISA-A2	≥30%	0.89 (0.79-0.98)	0.99 (0.97-1.00)	0.87 (0.78-0.97)
ELISA-B1	≥0.60	1.00 (1.00-1.00)	0.52 (0.47-0.57)	0.52 (0.47-0.57)
ELISA-B2 [b]	≥0.45	0.91 (0.82-0.99)	0.98 (0.96-0.99)	0.89 (0.80-0.97)
ELISA-B2 [b]	≥0.60	0.89 (0.79-0.98)	0.99 (0.99-1.00)	0.88 (0.79-0.97)
ELISA-C	≥0.50	0.93 (0.86-1.00)	0.94 (0.91-0.96)	0.87 (0.79-0.95)
IFAT-B	1:100	0.93 (0.86-1.00)	0.96 (0.94-0.98)	0.89 (0.81-0.97)
NAT	1:25	0.66 (0.52-0.80)	0.99 (0.97-1.00)	0.64 (0.50-0.78)

[a] ELISA-A1= competitive inhibition ELISA, VMRD Inc., WA, USA; ELISA-A2= competitive inhibition ELISA, VMRD Inc., WA, USA; ELISA-B1= indirect ELISA, Biovet Inc., St. Hyacinthe, Quebec, Canada; ELISA-B2= indirect ELISA, Biovet Inc., St. Hyacinthe, Quebec, Canada; ELISA-C= indirect ELISA, IDEXX Corp., Westbrook, ME, USA; IFAT-B= IFAT, USDA, Beltsville, MD, USA; NAT= *N. caninum* agglutination test, USDA, Beltsville, MD, US

[b] Inconclusive cut-off value, a 'suspicious' range is recommended to be used in between cut-off values 0.45 and 0.60, and for this reason, test characteristics for both cut-off values were established.

Table 3.3. Prevalence-adjusted, bias-adjusted kappa (PABAK), positive (P_{pos}) and negative (P_{neg}) test agreement values[a], with bold printed numbers indicating almost perfect agreement, on sera from 397 dairy cows.

Test [b]		ELISA-A1	ELISA-A2	ELISA-B1	ELISA-B2[c]	ELISA-C	IFAT-B	NAT
IFAT-A	PABAK	**0.94**	**0.95**	0.14	**0.96**	**0.87**	**0.91**	**0.90**
	P_{pos}	**0.87**	**0.89**	0.34	**0.92**	0.76	**0.83**	0.74
	P_{neg}	**0.98**	**0.99**	0.68	**0.99**	**0.96**	**0.98**	**0.97**
ELISA-A1	PABAK	-	**0.98**	0.15	**0.97**	**0.88**	**0.88**	**0.88**
	P_{pos}		**0.96**	0.35	**0.92**	0.78	0.77	0.70
	P_{neg}		**0.99**	0.69	**0.99**	**0.97**	**0.97**	**0.97**
ELISA-A2	PABAK		-	0.18	**0.98**	**0.88**	**0.89**	**0.88**
	P_{pos}			0.34	**0.94**	0.78	0.79	0.69
	P_{neg}			0.68	**0.99**	**0.97**	**0.97**	**0.97**
ELISA-B1	PABAK			-	0.13	0.23	0.18	0.06
	P_{pos}				0.32	0.45	0.39	0.25
	P_{neg}				0.68	0.71	0.69	0.66
ELISA-B2	PABAK				-	**0.88**	0.91	**0.90**
	P_{pos}					0.78	**0.81**	0.75
	P_{neg}					**0.97**	**0.97**	**0.97**
ELISA-C	PABAK					-	**0.84**	**0.80**
	P_{pos}						0.74	0.59
	P_{neg}						**0.95**	**0.94**
IFAT-B	PABAK						-	**0.83**
	P_{pos}							0.63
	P_{neg}							**0.95**

[a] PABAK, P_{pos}, P_{neg} interpretation: <0.2 slight agreement, 0.2 to 0.4=fair agreement, 0.4 to 0.6 = moderate agreement, 0.6 to 0.8 = substantial agreement, and >0.8 = almost perfect agreement (Dohoo et al., 2003)

[b] IFAT-A=IFAT, VMRD, Pullmann, WA, USA; ELISA-A1= competitive inhibition ELISA, VMRD Inc., WA, USA; ELISA-A2= competitive inhibition ELISA, VMRD Inc., WA, USA; ELISA-B1= indirect ELISA, Biovet Inc., St. Hyacinthe, Quebec, Canada; ELISA-B2= indirect ELISA, Biovet Inc., St. Hyacinthe, Quebec, Canada; ELISA-C= indirect ELISA, IDEXX Corp., Westbrook, ME, USA; IFAT-B= IFAT, USDA, Beltsville, MD, USA; NAT= *N. caninum* agglutination test, USDA, Beltsville, MD, US

[c] Cut-off value used: S/P ratio ≥0.6

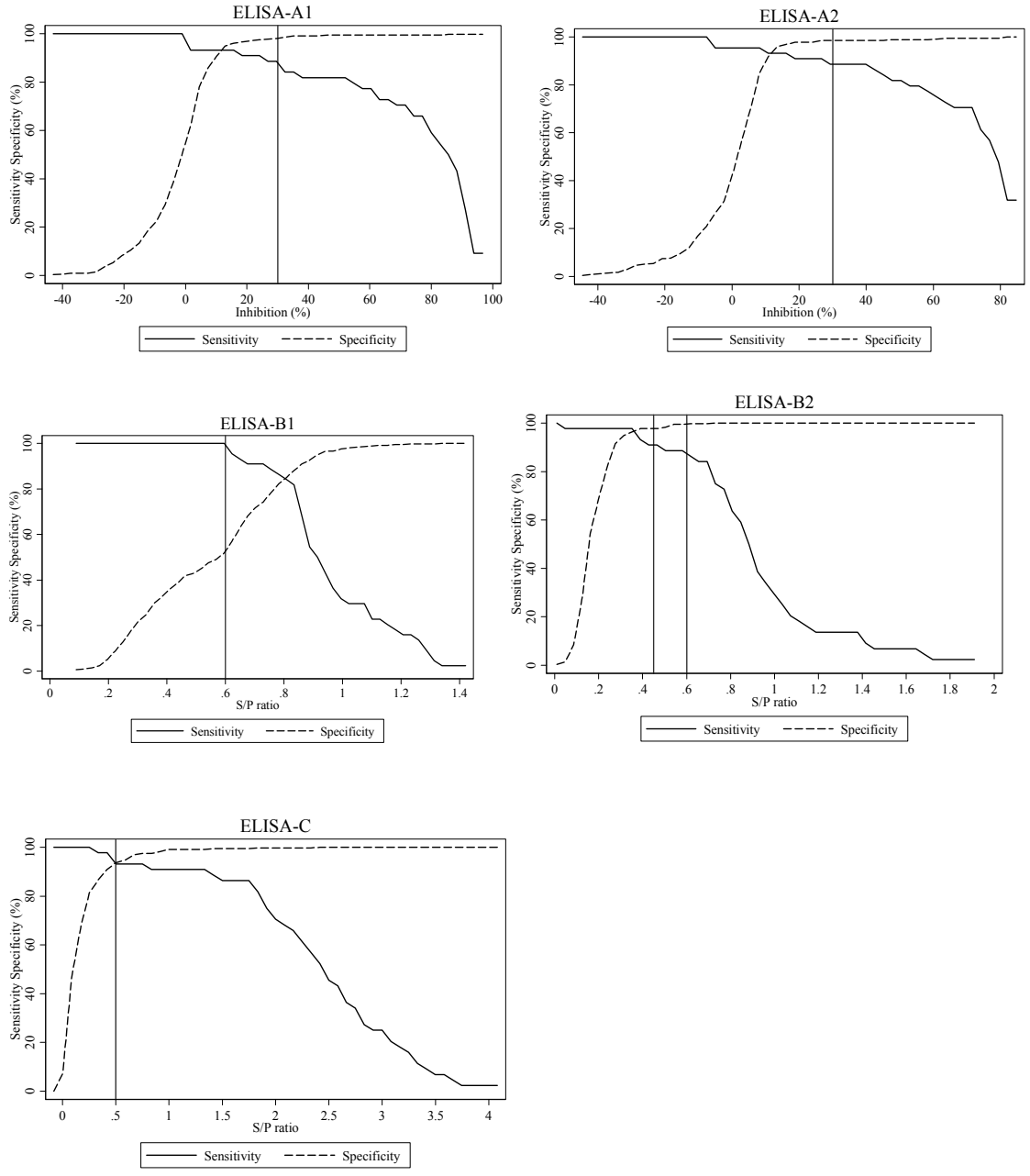

Figure 3.1. Two-graph receiver operating characteristic (TG-ROC) plots for five *Neospora caninum* ELISAs (ELISA-A1, ELISA-A2, ELISA-B1, ELISA-B2, ELISA-C)[a] compared to IFAT-A, with vertical line indicating cut-off value recommended by the manufacturer, on sera from 397 dairy cows.

[a] ELISA-A1= competitive inhibition ELISA, VMRD Inc., WA, USA; ELISA-A2= competitive inhibition ELISA, VMRD Inc., WA, USA; ELISA-B1= indirect ELISA, Biovet Inc., St. Hyacinthe, Quebec, Canada; ELISA-B2= indirect ELISA, Biovet Inc., St. Hyacinthe, Quebec, Canada; ELISA-C= indirect ELISA, IDEXX Corp., Westbrook, ME, USA

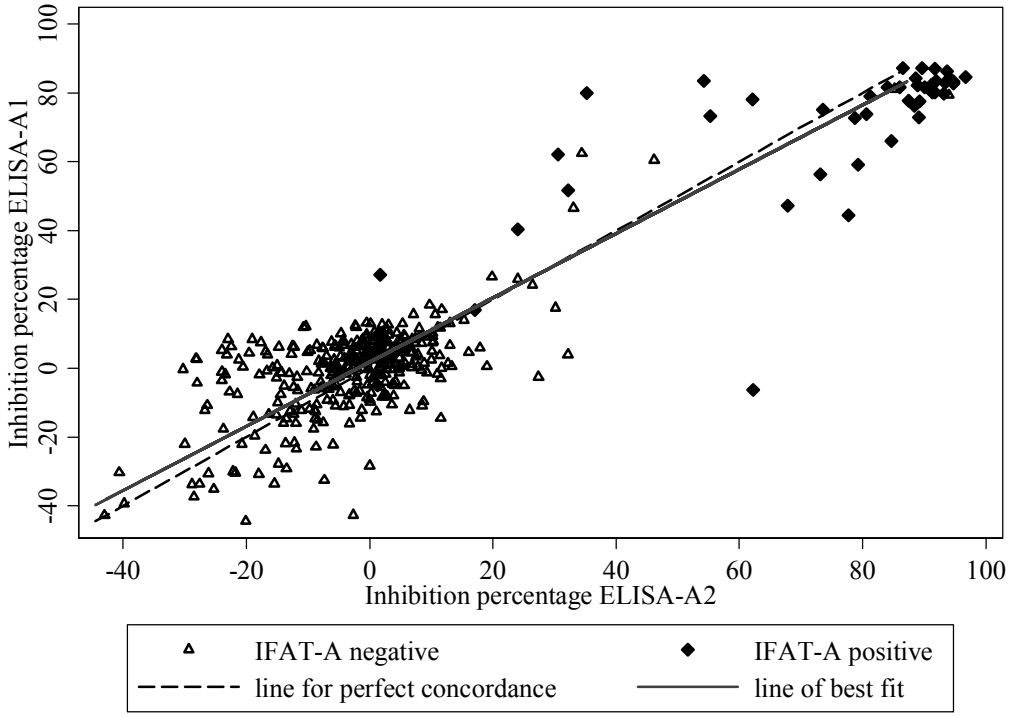

Figure 3.2. Concordance correlation plot of a competitive *Neospora caninum* ELISA (ELISA-A1 and ELISA-A2) carried out at two different laboratories 2 years apart on sera from 397 dairy cows.[a]

[a] IFAT-A= IFAT, VMRD Inc.Pullman, WA, USA; ELISA-A1= competitive inhibition ELISA, VMRD Inc., WA, USA; ELISA-A2= competitive inhibition ELISA, VMRD Inc., WA, USA

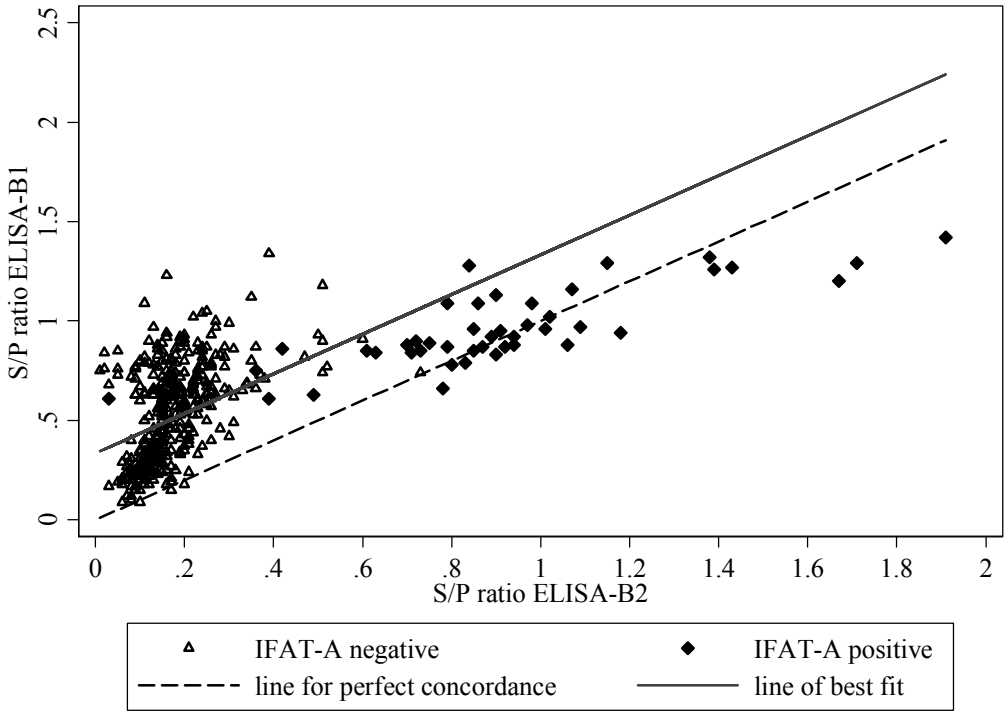

Figure 3.3. Concordance correlation plot of an indirect *Neospora caninum* ELISA (ELISA-B1 and ELISA-B2) carried out at two different laboratories 6 years apart on sera from 397 dairy cows[a]

[a] IFAT-A= IFAT, VMRD Inc.Pullman, WA, USA; ELISA-B1= indirect ELISA, Biovet Inc., St. Hyacinthe, Quebec, Canada; ELISA-B2= indirect ELISA, Biovet Inc., St. Hyacinthe, Quebec, Canada

4. THE EFFECT OF HEMOLYSIS ON THE PERFORMANCE OF TWO *NEOSPORA CANINUM* ENZYME-LINKED IMMUNOSORBENT ASSAYS USING CATTLE AND FOX SERA

4.1. Introduction

Neospora caninum is the most frequently diagnosed cause of abortion in cattle worldwide. Different wildlife species have been investigated as potential intermediate or definitive host (Gondim et al., 2004a; Gondim et al., 2004b; Lindsay et al., 1996; Schares et al., 2002). Antibody detection by serology is commonly used as a first method of screening wildlife for *N. caninum*. However, investigating wildlife is challenged by difficulties of sample collection. Samples are often derived from carcasses showing varying degrees of decomposition and the level of hemolysis of serum from decomposed animals may be highly variable. The question arises whether these sera are still appropriate for serological testing, because the reliability and performance of immunoassays may be affected (Lillehaug et al., 2003). Some sampling and storage factors may artificially induce hemolysis, such as prolonged storage of the blood before separation, rapidly forcing blood through small needles, excessive agitation when mixing, the physical act of centrifugation and separation of serum, or an improper storage temperature (O'Neill et al., 1989).

Although several seroprevalence studies for *N. caninum* in wildlife have been published (Almeria et al., 2002; Canon-Franco et al., 2004; Ferroglio et al., 2001; Ferroglio et al., 2003; Jakubek et al., 2001; Vianna et al., 2005), scarce information is available on the quality of *N. caninum* antibodies in animal blood with different degrees

of hemolysis. An experiment, using an indirect *N. caninum* immunostimulating complex enzyme-linked immunosorbent assay (ELISA) with cattle sera, showed that specific IgG antibodies were stable in sterile blood and decomposed lung tissue when stored at room temperature for up to 118 days (Jakubek et al., 2005). A reduced specificity was observed using severely hemolysed serum from 19 mountain lions in a *N. caninum* modified agglutination test (Packham et al., 1998). The significance of using non-hemolysed serum for antibody analysis, especially when dealing with wildlife, is important prior knowledge for adequate data collection.

The aim of this study was to evaluate the effect of hemolysis, as encountered in the field with wildlife and cattle sera, on the performance of two commercially available ELISAs frequently used in North America.

4.2. Materials and methods

4.2.1. Sample collection

Between August and October 2004, blood samples were collected from 10 randomly selected juvenile silver foxes (*Vulpes vulpes*) which were raised and kept at the Nova Scotia Agricultural College, Canadian Centre for Fur Animal Research, Nova Scotia, Canada. At least three generations of these presumed *N. caninum*-negative foxes were raised in wire cages and did not have any contact with tissue that could have been contaminated with *N. caninum*. Blood was taken from the cephalic vein, using vacutainer tubes without additives. After this initial blood collection, all 10 foxes were vaccinated subcutaneously with a commercially available bovine *N. caninum* vaccine (Neoguard®, Intervet, Whitby, ON, Canada). Blood was collected again from all 10 animals 3 and 6

weeks after vaccination, providing 30 fox blood samples in total. This vaccination induced an immune response to *N. caninum* and served as a positive control for test validation.

In September 2005, blood samples were also collected from 111 dairy cows in 3 *N. caninum*-positive herds on Prince Edward Island, Canada. The samples were obtained by puncture of the coccygeal vein using vacutainer tubes without additives.

4.2.2. Preparation of hemolysed and non-hemolysed samples

Approximately 5 h after collection, vacutainer tubes with 10 ml of blood were centrifuged for 10 min at 3000 x g. Non-hemolysed serum (1 ml) was transferred into a new collection tube and stored in a -20ºC freezer. To induce hemolysis, the vacutainer tubes with remaining blood were then vortexed for 20 s and stored at room temperature for 24 h. Subsequently, 1 ml of hemolyzed serum was transferred into a new collection tube, without prior centrifugation. The 60 fox samples and 222 cow samples were stored at -20ºC until testing, to ensure hemolysis and to preserve antibodies.

4.2.3. Laboratory analyses

4.2.3.1. Competitive ELISA

A monoclonal antibody-based competitive inhibition ELISA (cELISA, VMRD® Inc., Pullman, WA, USA) was used for the detection of *N. caninum* antibodies. *N. caninum* antibodies in a serum sample inhibit the binding of (horseradish peroxidase-labeled) *N. caninum* specific monoclonal antibody to *N. caninum* antigen coated on the plastic wells (Baszler et al., 2001). The cELISA is commercially available for use in cattle and goats.

The assay was performed according to the manufacturer's instructions (VMRD, 2006). No modifications were carried out when using fox sera, as no species-specific conjugate is necessary with this competitive inhibition technique. The 60 fox sera and 222 cattle sera (30 fox and 111 cattle sera were hemolysed) were analyzed with this cELISA.

To investigate whether serum dilution altered test characteristics observed with this cELISA, a subset of fox sera (7 *N. caninum*-negative and 8 *N. caninum*-positive controls) was used in a 1:20 serum dilution experiment. The cut-off value (30% inhibition was considered positive for *N. caninum*) recommended by the manufacturer for use in cattle was also used for foxes. Because of limited availability of test kits, only 15 sera and an arbitrarily chosen serum-dilution were used.

4.2.3.2. Indirect ELISA

As a comparison to the cELISA, a commercially available indirect ELISA (iELISA, Biovet® Inc., St. Hyacinthe, Quebec, Canada) was also used to analyze fox sera. The wells are coated with antigens of a *N. caninum* tachyzoite sonicated lysate (strain NC-1). The iELISA is for use with bovine serum. The assay was performed according to the manufacturer's recommendations (Biovet Inc., 2006), except the conjugate provided in the kit was replaced by a rabbit anti-dog IgG conjugate (Jackson ImmunoResearch Laboratories Inc., West Grove, USA). It was expected that these canine antibodies would respond to fox IgG (Chapter 6). Serial dilutions with reference sera from 10 foxes indicated that a serum dilution of 1:200 (as recommended for bovine sera) for foxes was appropriate (data not shown). Previous likelihood ratio analyses resulted in an S/P ratio of 0.19 as the optimal cut-off value when using fox sera (Chapter 6). Samples from some

animals were exhausted during repeated testing, and thus, only 10 *N. caninum*-negative and 15 *N. caninum*-positive fox sera were analyzed with the iELISA. Financial constraints limited the use of this assay to fox samples only, no cattle samples were analysed with this iELISA.

4.2.4. Statistical analyses

Sensitivity (Se) and specificity (Sp) for the two ELISAs could be determined based on 20 fox sera (10 *N. caninum*-negative pre-vaccination sera and 10 *N. caninum*-positive sera collected 6 weeks post-vaccination) because the "true status" of these two groups of foxes was known. For cattle, there was no information on the true status of the animals. The Se and Sp were calculated for both the non-hemolysed and hemolysed fox sera.

For both the cELISA and the iELISA, a Student's *t*-test was used to determine the significance of the difference between the hemolysed and non-hemolysed samples. Agreement between non-hemolysed and hemolysed test results for foxes and cattle was calculated for each ELISA using Lin's concordance correlation coefficient (CCC) (Lin, 1989). The CCC for the cELISA was based on 10 *N. caninum*-negative and 20 *N. caninum*-positive fox samples and 111 cattle field sera. For the iELISA, 10 *N. caninum*-negative and 15 *N. caninum*-positive fox control samples were used to calculate the CCC. Because agreement between hemolysed and non-hemolysed samples was the main interest for using the concordance correlation coefficient, it was appropriate to also include the repeated samples from *N. caninum*-positive foxes. Stata version 8 (Stata Corporation, College Station, TX, USA) was used for the statistical analyses of the data.

4.3. Results

4.3.1. Competitive ELISA

For the non-hemolysed fox sera, a Se of 1.00 (95% CI: 0.69-1.00) and a Sp of 1.00 (95% CI: 0.69-1.00) were calculated. For the hemolysed fox sera, a Se of 1.00 (95% CI: 0.69-1.00) and Sp of 0.50 (95% CI: 0.19-0.81) were calculated. The CCC between the non-hemolysed and hemolysed fox samples was 0.67 (95% CI: 0.50-085, Fig. 4.1). The inhibition percentages were lower for non-hemolysed sera compared to hemolysed sera from *N. caninum*-negative foxes ($p<0.01$). For the *N. caninum*-positive sera, there was no difference between non-hemolysed and hemolysed sera ($p=0.11$).

Dilution (1:20) of the fox-sera reduced inhibition percentages for *N. caninum*-negative samples below the recommended cut-off value of 30% inhibition for the cELISA. However, this dilution step decreased inhibition percentages of all samples tested considerably, and consequently resulted in a minimal sensitivity of 0 (95% CI: 0.00-0.53) and hence false-negative results. The specificity was 1.00 (95% CI: 0.59-1.00).

When analyzing the non-hemolysed cattle serum samples, 25 of 111 (22.5%) sera were *N. caninum*-positive. The CCC between the non-hemolysed and hemolysed cattle samples was 0.97 (95% CI: 0.95-0.98, Fig. 4.2). Inhibition percentages of non-hemolysed and hemolysed cattle sera were not significantly different ($p=0.25$).

4.3.2. Indirect ELISA

For the non-hemolysed fox sera, a Se of 1.00 (95% CI: 0.69-1.00) and Sp of 1.00 (95% CI: 0.69-1.00) were calculated. For the hemolysed fox sera, a Se of 0.80 (95% CI: 0.55-1.00) and Sp of 0.90 (95% CI: 0.44-0.98) were calculated. The CCC between the

non-hemolysed and hemolysed fox samples was 0.86 (95% CI: 0.77-95, Fig. 4.3). S/P ratios were higher in non-hemolysed compared to hemolysed *N. caninum*-positive fox sera ($p<0.01$), but tended to be lower for the *N. caninum*-negative sera ($p=0.08$).

4.4. Discussion

When analyzing hemolysed fox sera with the competitive ELISA, a significant decrease in Sp was observed versus non-hemolysed fox sera. Poor agreement was also observed between the results of non-hemolysed and hemolysed fox sera using the cELISA. This is illustrated by deviations from the line of perfect concordance in Figure 4.1. In contrast, using the same ELISA, the antibody titers in hemolysed bovine sera correlated well with the non-hemolysed duplicates (CCC=0.97). Results were only minimally influenced by the quality of the sample and there was a good separation between high and low inhibition results (Fig. 4.2).

There is no known reason why hemolysed fox sera would behave differently in a cELISA compared to hemolysed cattle sera. Fox and cattle blood samples were treated in the same manner to artificially produce hemolysed sera, thus excluding this procedure as a possible explanation for the observed differences in results between species. Because inhibition percentages of *N. caninum*-negative samples were high for the hemolysed samples, 5 out of the 10 negative samples lead to false-positive results. It is unlikely that hemolysed samples contained more *N. caninum*-antibodies than non-hemolysed samples, and it is more likely that certain proteins or glycoproteins in these hemolysed sera were bound non-specifically to the *N. caninum* antigen coated on the plastic wells, thereby preventing attachment of the secondary competing antibody.

In an attempt to improve performance of this cELISA in hemolysed fox sera, a dilution experiment was performed. There was a significant loss in Se, thus, diluting serum does not appear suitable for the cELISA. However, a lower dilution with an adjustment in the inhibition percentage cut-off value may be more appropriate than the 1:20 dilution that was evaluated in this study.

The iELISA performed more consistently (Se=0.8 and Sp=0.9) than the cELISA (Se=1.0 and Sp=0.5) when comparing non-hemolysed and hemolysed fox sera. The CCC of 0.86 for the iELISA indicated reasonable correlation between non-hemolysed and hemolysed fox sera. Non-hemolysed *N. caninum*-positive sera had significantly higher S/P-ratios than the hemolysed samples. In addition, sample preparation could have contributed to a decrease in antibodies, because hemolysed serum was taken from the vacutainer tube after 1 ml of non-hemolysed serum was collected. By removing 1 ml of serum with *N. caninum* antibodies, the remaining hemolysed serum may have contained a lower concentration of antibodies.

The dilution factor required in this iELISA was 1:200 for cattle. The same dilution for foxes resulted in best possible test characteristics to discriminate *N. caninum*-positive and *N. caninum*-negative samples. This dilution factor may be an explanation for the superior performance of the iELISA compared to the cELISA. By diluting, less hemolysis products may have interfered with antibody attachment. The decrease in S/P-ratio for hemolysed samples observed in the iELISA has to be considered when analyzing hemolysed serum but could potentially be controlled by adjusting the cut-off value.

Other studies have found similar results when determining the effect of hemolysis on test performance of various substances in different species. Hemolysis significantly

decreased the measured concentration of haptoglobin in porcine serum samples. This was possibly explained by the ability of haptoglobin to form very stable complexes with free haemoglobin, resulting in a decreased binding of haptoglobin antibody used in this ELISA (Petersen et al., 2001). The addition of haemolysate to serum samples from dogs, did not have an influence on cortisol levels, but showed a significant effect when measuring free thyroxine by ELISA. The differences were unrelated to the amount of haemoglobin added, suggesting that other red cell constituents were interfering with the free thyroxine assay (Lucena et al., 1998).

A significant difference in performance of two ELISAs, using hepatitis and human immunodeficiency virus antigens, was observed when using grossly hemolysed cadaveric serum (Novick et al., 1996). A study evaluating serological screening of cadaveric human sera for donor selection showed that the accumulation of interfering hemolysis/autolysis products after death seemed to be highly variable and not directly related to the time elapsed between death and collecting cadaveric serum (Heim et al., 1999). The prosthetic group that mediates reversible binding of oxygen by haemoglobin, is a porphyrin that may adhere to microwells and oxidize the substrate solution, causing a color change in ELISA. However, three ELISAs were effective in screening cadaveric sera for antibodies against human immunodeficiency virus (Pepose et al., 1987), despite the level of hemolysis. Human sera stored for 48 hours at room temperature had decreased levels of IgG1, whereas other IgG subclasses remained unchanged (Maninger et al., 1996).

In our study, we did not measure the degree of hemolysis in detail, as has been performed in other studies (Lopez et al., 1989; Lucena et al., 1998; Martinez-Subiela et al., 2002). This may have given more insight to the cause of the decreased performance

observed. However, the degree of sample hemolysis was not correlated with ELISA activity in a study evaluating sample hemolysis as a cause of false-positive reactions in ELISA for feline leukemia virus antigen (Lopez et al., 1989). Investigating in more detail which aspects of degradation of a blood sample are responsible for the poorer performance observed in hemolysed samples, may explain the results obtained in this study.

The immune response and therefore *N. caninum*-antibody levels in the positive control samples created by vaccinating foxes as performed in this study may be different from antibody levels in naturally infected *N. caninum*-positive samples. The high Se in both assays when using non-hemolysed sera from vaccinated foxes, demonstrates that the antibody response created after vaccination, was consistently identified as *N. caninum*-positive by both assays. This illustrates a convenient opportunity to create *N. caninum*-positive control samples without experimentally infecting animals with *N. caninum*.

In conclusion, the cELISA has the advantage of not being species-specific, and performs well with non-hemolysed sera from cattle (Baszler et al., 2001) and foxes, as shown in this study. However, caution is required when using this cELISA with hemolysed fox sera. The iELISA produced better test performance characteristics with the hemolysed fox sera used in this study. With appropriate cut-off determination and conjugate, the iELISA was found to be the preferred test to use when working with hemolysed fox sera.

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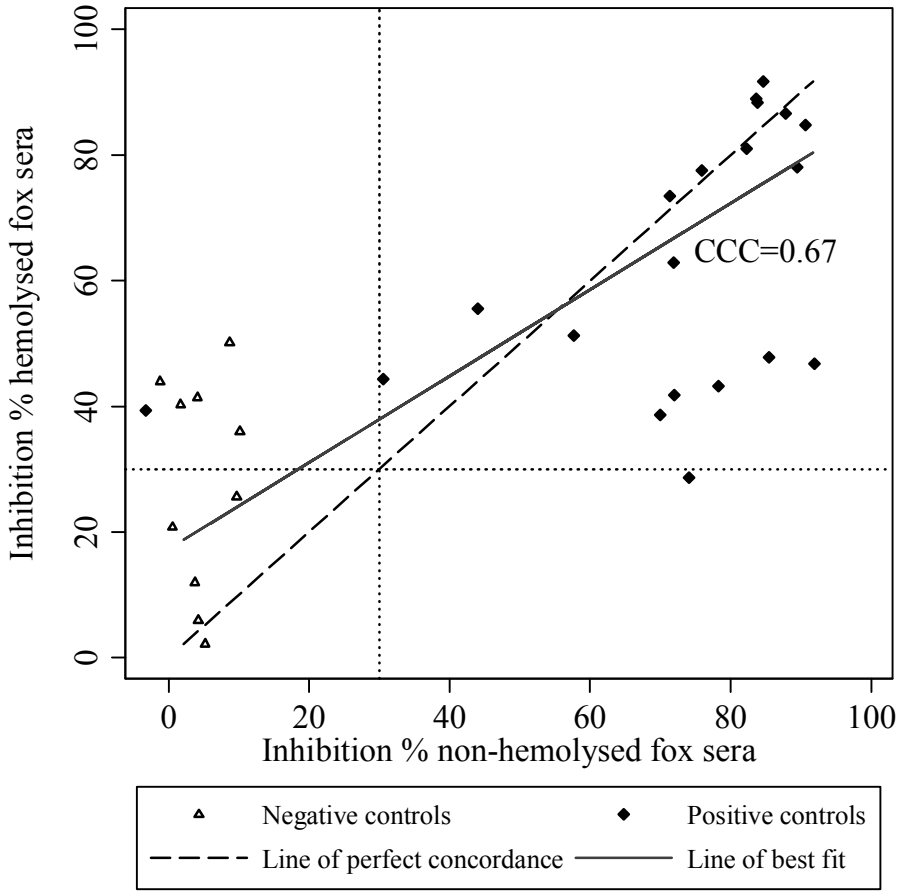

Figure 4.1. Concordance correlation between 30 non-hemolysed and hemolysed sera from 10 juvenile silver foxes (*Vulpes vulpes*), as measured by the competitive *Neospora caninum* ELISA. The vertical and horizontal dotted lines (••••) indicate a cut-off value at 30% inhibition.

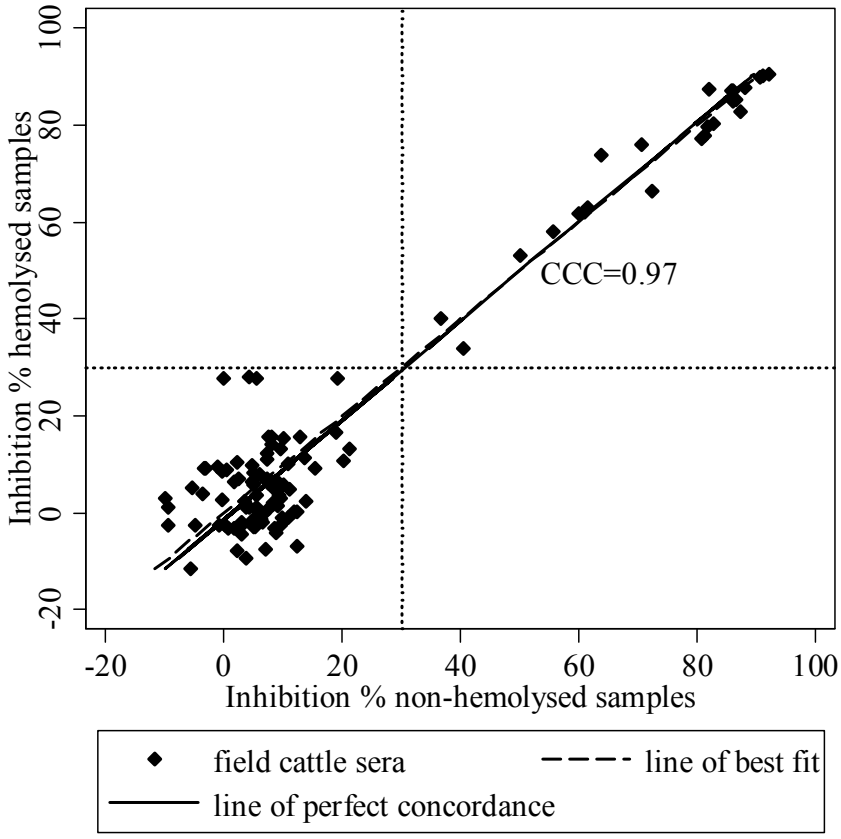

Figure 4.2. Concordance correlation between non-hemolysed and hemolysed sera from 111 dairy cows on Prince Edward Island in 2004, as measured by the competitive *Neospora caninum* ELISA. The vertical and horizontal dotted lines (••••) indicate a cut-off value at 30% inhibition.

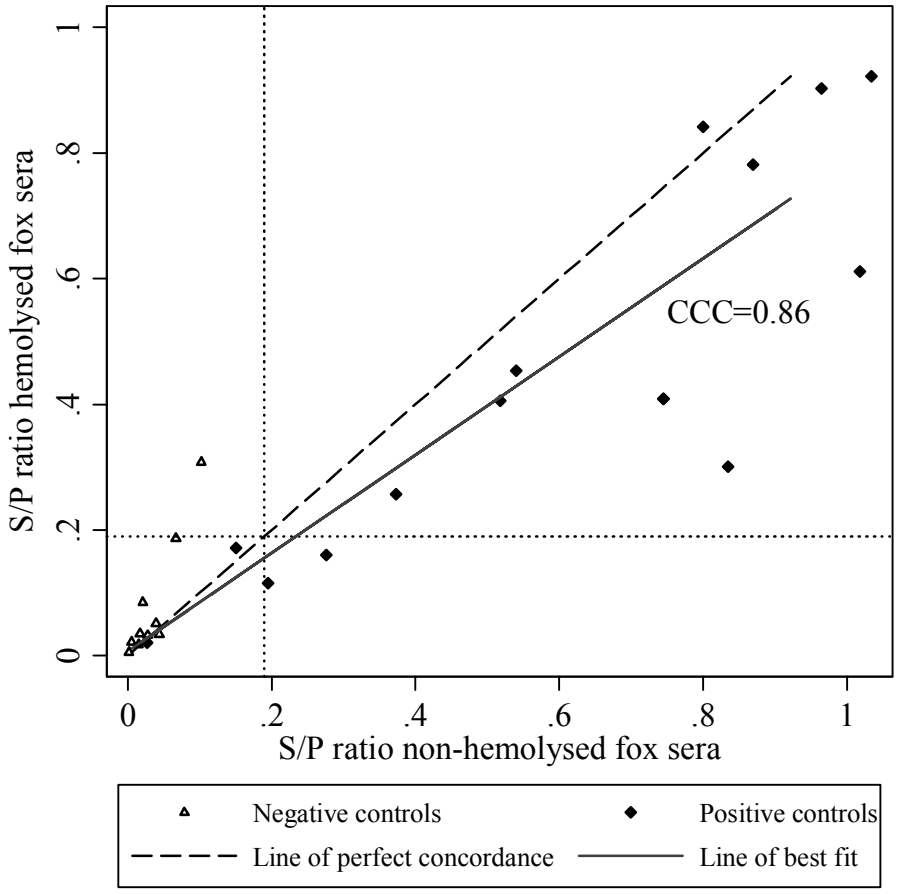

Figure 4.3. Concordance correlation between 25 non-hemolysed and hemolysed sera from 10 juvenile silver foxes (*Vulpes vulpes*), as measured by the indirect *Neospora caninum* ELISA. The vertical and horizontal dotted lines (••••) indicate a cut-off value S/P ratio of 0.19.

5. POPULATION STRUCTURE AND HABITAT OF THE WILD FOX (*VULPES VULPES*) AND COYOTE (*CANIS LATRANS*) ON PRINCE EDWARD ISLAND, CANADA

5.1. Introduction

The Canadian province of Prince Edward Island (PEI) consists mainly of agricultural and forested land. Hunting and trapping are traditional outdoor activities on PEI. Trappers and hunters use dead domestic stock to attract foxes and coyotes to a given area. In the 2004-2005 hunting and trapping season, 809 foxes and 343 coyotes were harvested, which was a 23% and 28% reduction in harvest for foxes and coyotes, respectively, compared to the previous season (R. Dibblee, unpublished data). It is estimated that the density of foxes and coyotes in the province is 1 and 0.4 animals / km^2, respectively (personal communication, R. Dibblee). A similar density has been described for coyotes in the neighbouring province New Brunswick (Dumond and Villard, 2000).

The coyote population on PEI has increased rapidly since the first occurrence in 1983 and occurs throughout the island. The successful expansion of coyotes is attributed to their ability to adapt to various habitats, as well as to exploit a variety of food sources including small mammals, ungulates, carrion and human garbage (Parker, 1995). Coyotes are sighted close to cattle, poultry and hog farms, where they scavenge dead cattle, poultry or pigs. The role of coyotes as predator of domestic livestock is also frequently mentioned as a reason for controlling this species (Chambers, 1992; Mitchell et al., 2004; Parker, 1995). Furthermore, *Neospora caninum* is a frequent cause of abortion in cattle, and can be transmitted to cows via feed contaminated with feces from coyotes containing

this parasite (Gondim, 2006).

Many foxes have lost their fear for humans as they are often fed on campgrounds and in urban areas. Their loss of fear for humans and their invasion into urban areas may pose a risk for human health. In Canada, foxes are carriers of diseases like rabies (Adkins and Scott, 1998) and hydatid disease (*Echinococcus* spp.) (Eckert et al., 2000). These diseases can have substantial public health and economic implications, yet rabies nor *Echinococcus* spp. are not known to be present on PEI. Feces from foxes (and coyotes) may contain other zoonotic parasites, for example *Toxocara canis* (Smith et al., 2003). Furthermore, foxes may also play a role in disease transmission, such as *N. caninum*, to livestock (Wapenaar et al., 2006).

In eastern Canada, several studies have documented specific aspects of coyote demography. These studies performed in Quebec, Nova Scotia and New Brunswick focused on body condition score (Dumond et al., 2000; Poulle et al., 1995), productivity (Jean and Bergeron, 1984) and social organization and space use (Patterson and Messier, 2001). Two studies were performed on PEI (Field, 2003; Gautreau, 2004), however, only a small number of animals were studied. To validate findings on the role of coyotes on PEI from these previous studies, data on more animals are required. Very few studies on foxes were described for eastern Canada. One report from PEI is available describing the red fox population in 1972 to 1980 (Curley et al., 1983). Long distance movement (170 km) of one red fox was reported in Ontario (Rosatte, 2002) and a description of home range and habitat associations of two social groups of red foxes was performed in suburban Toronto, Ontario (Adkins and Scott, 1998). However, the demography of foxes on PEI may well have changed after 1980 and is expected to be different from a suburban

environment such as Toronto. An accurate and up to date description of the fox and coyote population structure and dynamics on PEI is required to investigate the risk of this animal in transmitting diseases to domestic animals and humans. The objective of this study was to gain an understanding of the population structure and habitat of the wild fox and coyote on PEI.

5.2. Materials and methods

5.2.1. Field sample collection

Thirty-two registered fox and coyote trappers and hunters from across PEI were contacted and agreed to participate in this study. All fox and coyote carcasses were obtained via the hunters and trappers during their hunting and trapping activities from 19th October 2004 until 24th March 2005. No foxes or coyotes were killed deliberately for this study.

All carcasses were sampled as soon as possible after death, varying from a few hours to 5 days. Most carcasses were sampled within 24 hours after death at the location where they were skinned and stored. Sex, date of death, and location where the animal was killed were recorded. A canine tooth of the lower jaw was collected for age analysis. To differentiate between a juvenile and an adult animal, radiographs were taken of the individual canine teeth to assess the width of the pulp cavity. In general, juveniles are the young of the year, however, as the study period was in fall and winter, the juveniles in our study were at least 6 months old. The age of adult animals was determined by counts of the annual growth zones in the canine tooth cementum (Johnston Biotech, Sarnia, Ontario, Canada) (Grue and Jensen, 1976; Johnston et al., 1987). Since most foxes and

coyotes give birth in spring (Parker, 1995) and sample collection was performed in fall and winter, the minimum age recorded was 0.5 years and increased in 1 year intervals.

Reproductive tracts were removed from female fox and coyote carcasses. Reproductive status was assessed as the pregnancy rate based on the presence or absence of placental scars in females older than 0.5 year of age. Dark placental scars were considered in the estimation of litter size of the most recent breeding season; pale scars may be from the previous reproductive season (Martorell Juan et al., 1993).

5.2.2. Habitat characteristics

Location of where the animals were killed was recorded on a map. Latitude and longitude of these locations were recorded using an interactive mapping system (http://www.gov.pe.ca/mapguide). Each animal was identified by its latitude and longitude using a commercial GIS software program (Arc View GIS 9, Redlands, CA). The map was projected into Universal Transverse Mercator 1983 (UTM83), Zone 20 units. The home ranges of foxes and coyotes were assumed to be 9 km^2 (Lloyd, 1980) and 50 km^2, respectively, based on previous studies (Field, 2003; Gautreau, 2004; Hidalgo-Mihart et al., 2006). In order to determine the predominant land use type within these home ranges, land use data were utilized from field-validated aerial photographs of PEI taken in 2000. The data were redefined into four general land use categories: forest, agriculture, wetland and urban areas. To describe the habitat characteristics of the collected animals, percentages of land use within the assumed home ranges of individual foxes and coyotes were calculated.

5.2.3. Statistical analyses

A one-sample proportion test was used to determine differences in sex and age for each species. This test was also used to compare habitat proportions for each species' home range with the overall land use distribution on PEI. Chi-square analysis on contingency tables was used to investigate distribution of age, sex and reproductive status. A Students' T-test was used to investigate distribution of litter size and age. Stata version 8 (Stata Corporation, College Station, TX, USA) was used for the statistical analyses of the data. Statistical significance was defined at $p<0.05$.

5.3. Results

5.3.1. General descriptive data

Information was collected on 472 wild canids, comprised of 271 foxes and 201 coyotes, being 33.5 and 58.6% of the foxes and coyotes harvested in the 2004-2005 trapping and hunting season on PEI. The method of kill for most foxes (97%, n=261) and coyotes (57%, n=111) was trapping. Five foxes and 2 coyotes were collected after road-kill and the remaining foxes (n=4) and coyotes (n=82) were killed by hunters. Foxes were mainly collected in December; coyotes were collected more consistently throughout the hunting and trapping season (October-March). Coyote collection was evenly distributed across PEI, whereas fox collection was mainly clustered in central PEI (Fig. 5.1). A high number of foxes (35%) collected by one trapper contributed to this clustering. Sex was recorded for 426 animals: 115 female and 131 male foxes, and 92 female and 88 male coyotes. The sex ratio was not significantly different from 1:1 for foxes ($p=0.31$) and coyotes ($p=0.76$). Due to incomplete recording not all data was available for all carcasses

that were collected.

5.3.2. Age distribution and litter size

Canine teeth were collected from 271 foxes and 184 coyotes. The proportion of juvenile foxes (58%, n=158) was higher than the proportion of juvenile coyotes (48%, n=89) (p=0.04) (Table 5.1). The age distribution ranged from 0.5-13.5 years of age for both foxes and coyotes. The average age of the foxes was 1.5 years (95% CI: 1.3-1.7), but 2.1 years (95% CI: 1.7-2.4) for coyotes. Median age of foxes and coyotes was 0.5 and 1.5 years, respectively. Juvenile foxes were more often male than female (p=0.02, Table 5.1), while foxes ≥3.5 years old were more often female than male (p=0.05). Both associations, however, were not found for coyotes (p>0.90).

Placental scar counts were performed on 51 fox uteri and 32 coyote uteri from adult animals. Uteri were not available from all female foxes and coyotes, mainly due to severe autolysis of carcasses that made placental scar counts unreliable. Thirty-four of 51 (67%) adult female foxes and 22 of 32 (69%) coyotes were reproductively active. Foxes older than 1.5 years tended to be more often reproductively active than 1.5 year-old foxes (p=0.09). This trend was not observed in coyotes (p=0.21). However, in coyotes the sample size was small (n=32). Placental scars were not observed in any of the juvenile foxes and coyotes. Average litter size for foxes and coyotes was 5.0 (95% CI: 4.5-5.5) and 5.2 (95% CI: 3.9-6.4), respectively. Number of placental scars ranged from 0 to 7 in foxes and 0 to 11 in coyotes (Table 5.2). In coyotes, the litter size of primiparae was smaller than for multiparae (p=0.001). This increase in litter size with increasing age was not observed in foxes (p=0.36). The oldest fox was a 13.5 year old female, and she had

no dark or pale placental scars, indicating she had no litter in the most recent breeding season. Two coyotes of 13.5 years of age were caught, 1 male and 1 female coyote (with 5 placental scars).

5.3.3. Habitat characteristics

An exact location of the sources of animals was obtained for 266 foxes and 185 coyotes. Agriculture was the main habitat type (52%) comprising the home ranges of the foxes. For coyotes, forest was the most common habitat (44%) followed closely by agriculture (43%). The home ranges of collected foxes contained significantly higher percentages of urban (13%) and agriculture (52%) land use when compared with the land use distribution on PEI as a whole (Table 5.3). Proportion of wetland (6%) and forest (29%) were significantly lower in the home ranges of foxes when compared to the land use distribution on PEI. The home range of collected coyotes also contained a higher percentage of agricultural land use (43%), compared to the land use distribution on PEI (Table 5.3). Hunted coyotes had a higher percentage of agricultural land (47%) in their home ranges compared to trapped coyotes (40%). This was inverted for percentage forest in their home ranges; 40% of the home ranges of hunted coyotes and 47% of the home range of trapped coyotes consisted of forest (Table 5.3). However, this difference between trapped versus hunted coyote home ranges was not significant (p=0.17).

5.4. Discussion

This study describes the population and habitat of foxes and coyotes on PEI in fall and winter 2004-2005. The results of this study help to better determine the risk these

wild canids pose on humans and domestic livestock and can assist in defining control strategies. In general, an established population of coyotes that is not being exploited will have an equal sex ratio as observed in our study (Parker, 1995). A study in urban foxes in Bristol (UK) between 1977 and 1986 observed a higher ratio of male foxes (Harris and trewhella, 1988). Sampling methodology has been found a potential explanation for populations with male favoured sex ratios. In most mammalian populations, males move greater distances than females resulting in males being more susceptible to trapping.

Because most foxes and coyotes give birth in March and April (Parker, 1995), no animals younger than 6 months were likely to be present in this study. The high proportion of foxes (58%) that were juveniles may represent the actual fox age ratio but may also be caused by the fact that less experienced juvenile foxes are easier to trap. A previous study reported that juveniles composed 64% of a mostly trapped fox population studied on PEI during 1972 and 1980 (Curley et al., 1983), and a ratio of juvenile foxes of 75% was reported in a 1978 study from Southern Minnesota (Simon et al., 1981). The animals collected in this study were trapped and hunted, but the proportions were not reported. A high proportion of juvenile foxes in our study may also be attributed to the abundance of food; an increase in food supply would enable better body condition and survival rates for young individuals (Dumond and Villard., 2000). The average age of foxes (1.5 years), strongly influenced by the number of juvenile foxes, was significantly lower than the average age for coyotes (2.1 years). In eastern New Brunswick, Canada, an unusually high average age of 5.6 years was observed in coyotes (n=77) (Dumond and Villard, 2000). Coyotes under 3 years of age accounted for 85% of the population in 14 studies in North America (Parker, 1995). This is similar to 78% (144 of 184 coyotes) of

the population in our study. A small proportion of the sample consisted of very old animals, 1 fox and 2 coyotes of 13.5 years of age. A coyote of 16.5 years old was previously reported in New Brunswick (Dumond et al., 2000). However, to our knowledge, this is the first description of a wild fox as old as 13.5 years. Because of the scarcity of fox and coyote data available from PEI, it is not feasible to draw conclusions towards a growing or declining population. However, the presence of animals of this high age demonstrates long survival time, despite yearly harvesting.

The average litter size (5.0) of foxes observed in our study corroborates the findings from a previous study performed on PEI between 1972 and 1980, where a litter size of 5.2 was observed (Curley et al., 1983). A smaller mean litter size of 4.7 and 3.3 was observed in a studies on foxes around Bristol, UK (Harris et al., 1988) and North-eastern Spain (Martorell et al., 1993), respectively.

The percentage of reproductively active female coyotes of 69% is higher than previous findings on PEI (54%) (Field, 2003). In a study in New Brunswick, Canada, only 41% of adult female coyotes were reproductively active, and on average 6.6 placental scars were present in females older than 5 years (Dumond et al., 2000). The same average litter size of 6.6 and a pregnancy rate of 77% was observed in a coyote study in California (Sacks, 2005). It would appear that coyotes have responded to the persecution of hunting and trapping through compensatory reproduction (Chambers, 1992; Knowlton, 1972; Todd et al., 1983). Litter sizes of coyotes generally vary between 3 and 4 per litter in established populations, while litter sizes in harvested populations of coyotes vary between 5-8 pups per litter (Field, 2003). The litter size of coyotes in our study (5.2) was similar to a study in British Colombia (5.4) (Atkinson et al., 1991). In our study, an increasing litter size for

multiparae coyotes and increasing pregnancy rate for multiparae foxes was observed (Table 5.2).

The presence of placental scars is most commonly used in wildlife to indicate the number of young born in the most recent litter. However, counting placental scars is a crude measure of reproductive success because it takes no account of pup survival. A study in captive wild-caught coyotes describing an index of fetal viability found that 75-85% of the placental scars represented live pups in most years, although lower percentages were observed among younger and very old females (Green et al., 2002).

A previous study (Field, 2003) has shown that relative abundance of foxes and coyotes on PEI was uniform throughout the island and that the presence of coyotes is associated with the presence of foxes. Coyotes were collected in an even distribution over the island in our study, and we therefore feel confident to infer findings from these animals to the coyote population of PEI. The method of kill for 97% of the collected foxes was trapping, in contrast to 60% for coyotes, therefore the home range characteristics are considered as an accurate representation of the true habitat of foxes but may be less accurate for coyotes. Trapped animals were likely trapped in their home range. For the hunted animals, the home range location may not have been estimated accurately because some of these animals were tracked for a long period with dogs before being killed. This was confirmed by the different proportion of forest and agricultural land in the home ranges of trapped versus hunted coyotes (Table 5.3). However, the general habitat characteristics observed in this study corroborate findings from a previous study on PEI in 2001-2003 (Field, 2003). The home range size of coyotes varies considerably dependent on the region, availability of food, human activities, reproductive season and the technique to

calculate a home range (Atkinson et al., 1991; Field, 2003; Gautreau, 2004). In urban habitats, home ranges are small compared with those that occur in rural habitats (Atwood et al., 2004). Because this study was performed in a rural area in winter, a large home range estimate (50 km^2) was used.

For coyotes utilizing agricultural land, control efforts should be focused on limiting disease transmission to livestock by controlling contact of wild canids with livestock. Previous studies have shown that eradicating wild canids by bounty hunting is ineffective, due to their compensatory reproduction (Chambers, 1992; Parker, 1995). Coyote control should therefore involve making agricultural areas a less easy food supply. Proper disposal of dead stock, coyote-proof fencing and guard dogs or other animals, such as donkeys and llamas, are realistic methods to reduce contact between coyotes and livestock at farms (Smith et al., 2000).

The home range of foxes included a large part of agricultural land but also urban areas, giving them ample opportunity to transmit diseases, such as rabies, *Echinococcus* spp. or *Toxocara canis*, to humans and domestic animals in the vicinity. This drift to urban areas is likely related to food availability. With foxes approaching the urban areas, their control should be focused on limiting disease transmission to humans and domestic animals. Reducing the contact between humans and foxes is recommended in addition to reducing the food availability in urban areas. Public education, particularly concerning the approach and (non)-handling of wildlife and the vaccination of pets, are crucial if high densities of humans and foxes are to continue to coexist.

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Table 5.1. Distribution of age and sex in 246 foxes and 163 coyotes collected in 2004-2005 on Prince Edward Island, Canada[a].

	Fox				Coyote			
Age	Male		Female		Male		Female	
(years)	N	%	N	%	N	%	N	%
0.5	84	64	57	50	35	45	40	47
1.5	19	15	21	18	14	18	15	17
2.5	17	13	18	16	10	13	11	13
3.5	3	2	10	8	6	8	7	8
4.5	4	3	3	3	4	5	5	6
5.5-10.5	4	3	4	3	6	8	7	8
11.5-13.5	0	0	2	2	2	3	1	1
Total	131	100	115	100	77	100	86	100

[a]271 foxes and 184 coyotes were examined for age, while sex was recorded for 246 foxes and 180 coyotes.

Table 5.2. Age-related pregnancy rate, average litter size and number of placental scars found in 51 foxes and 32 coyotes collected in 2004-2005 on Prince Edward Island, Canada.

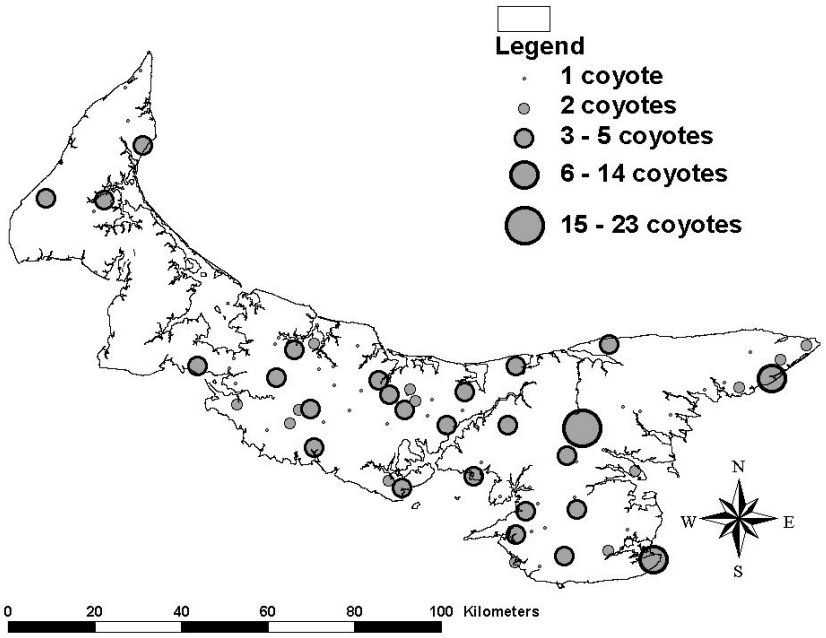
		Pregnancy	Average litter	Placental scars					
	Age (years)	rate (%)	size (n)	0	1-2	3-4	5-6	7-11	Total
Fox	1.5	50	4.9	8	0	4	3	1	16
	2.5	76	5.4	4	0	3	8	2	17
	3.5	70	4.7	3	1	2	3	1	10
	4.5-13.5	75	4.8	2	0	3	2	1	8
	Total	67	5.0	17	1	12	16	5	51
Coyote									
	1.5	55	2.5	5	3	3	0	0	11
	2.5	55	5.7	5	0	1	4	1	11
	3.5	100	7.0	0	0	1	1	1	3
	4.5-13.5	100	6.3	0	0	0	5	2	7
	Total	69	5.2	10	3	5	10	4	32

Table 5.3. Comparison of the distribution of land use on Prince Edward Island (PEI) and the home range of 266 foxes (*Vulpes vulpes*), 185 coyotes (*Canis latrans*) collected in 2004-2005 on Prince Edward Island, Canada. Home ranges of trapped (n=101) and hunted (n=79) are described in more detail.

	PEI	Home range foxes	Home range all coyotes	Home range trapped coyote	Home range hunted coyote
Forest (%)	47	29 [a]	44	47	40
Agriculture (%)	39	52 [a]	43	40	47 [b]
Wetland (%)	8	6	7	6	7
Urban (%)	6	13 [a]	6	7	6

[a]Significant ($P<0.01$) difference between the percentage of land use in the home range of foxes and the land use distribution on PEI.

[b]Trend ($P=0.07$) in difference between the percentage of land use in the home range of hunted coyotes and the land use distribution on PEI.



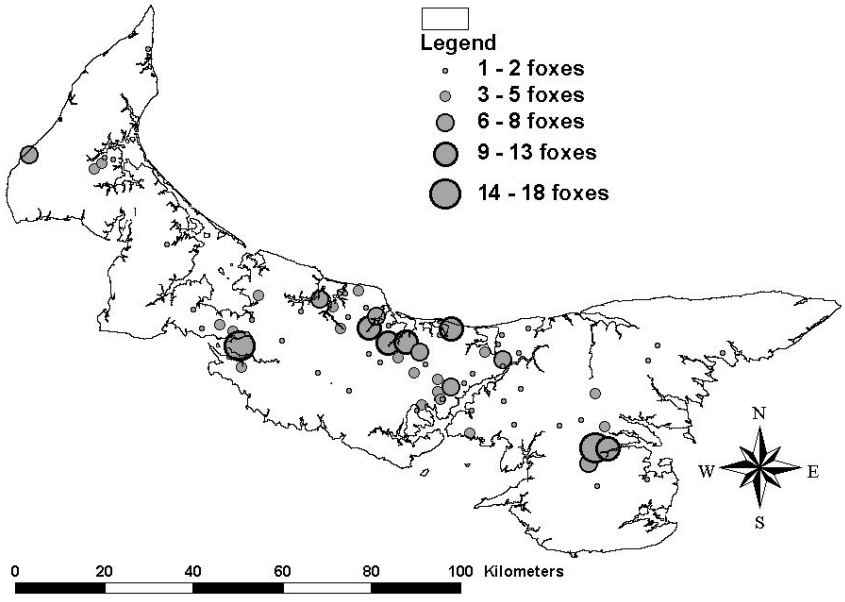


Figure 5.1. Graduated points of locations on Prince Edward Island, Canada where foxes or coyotes were trapped or hunted.

6. EVALUATION OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY TO DETERMINE THE SEROPREVALENCE OF *NEOSPORA CANINUM* IN FOXES (*VULPES VULPES*) AND COYOTES (*CANIS LATRANS*) ON PRINCE EDWARD ISLAND, CANADA

6.1. Introduction

Neospora caninum is the most frequently diagnosed cause of abortion in cattle worldwide (Dubey, 1999). Congenital infection appears to be the major mode of transmission in cattle but infection can also be caused by ingestion of oocysts shed by definitive hosts such as dogs and coyotes (Dijkstra et al., 2001; Dijkstra et al., 2002; Gondim et al., 2004a). A spatial association has been observed among abundance of wild canids, and seroprevalence of *N. caninum* in a population of beef calves in Texas (Barling et al., 2000). However, this association has not been confirmed for any other regions. In contrast, a risk factor study in Ontario found a negative association between 'the frequency of wild canids observed on premises' and the occurrence of *N. caninum*-related abortion (Hobson et al., 2005).

Serological evidence for *N. caninum* infection in wild canids has been documented (Canon-Franco et al., 2004; Hamilton et al., 2005; Lindsay et al., 2001; Simpson et al., 1997). For example, antibodies to *N. caninum* were detected in 5 (10%) of 52 coyotes (*Canis latrans*) in Texas (Lindsay et al., 1996) based on an indirect fluorescent antibody test (IFAT), and 4 (15.4%) of 26 gray foxes (*Urocyon cinereoargenteus*) from a non-agricultural area in South Carolina had low titres to *N. caninum* (Lindsay et al., 2001), determined by the direct agglutination test (DAT). Antibodies against *N. caninum* were also detected based on an IFAT in 12 (11%) of 113 coyotes (*Canis latrans*) from Utah,

Colorado and Illinois (Gondim et al., 2004b). However, the accuracy of these prevalence estimates is unknown because there are no reports evaluating the test performance of assays for antibody detection of *N. caninum* in wild canids.

Testing options for determining seroprevalence estimates of *N. caninum* in wild canids include the IFAT, DAT, and enzyme-linked immunosorbent assay (ELISA). An IFAT is the most commonly used assay in previous seroprevalence studies of *N. caninum* in wild canids (Barber et al., 1997; Gondim et al., 2004b; Lindsay et al., 2001). However, these studies typically collected only a limited number of samples. Alternatively, an ELISA is more rapid when dealing with large numbers of samples and is less dependent than IFATs on the experience of the diagnosticians performing the assay.

An indirect ELISA (Biovet Inc., St. Hyacinthe, Canada) was previously validated to perform satisfactorily with both hemolysed and non-hemolysed sera from foxes (Chapter 5). However, this study also reported evidence that non-hemolysed *N. caninum*-positive control samples from foxes had significantly higher S/P ratios compared to their hemolysed counterparts. Hemolysis is frequently observed when collecting serum from wild canids, especially when samples are collected post-mortem (unpublished observation). Therefore, an additional accuracy concern of serological tests (such as the ELISA for *N. caninum*, designed for bovine sera but utilized on wild canid sera) is that hemolysis may influence the performance of the diagnostic test (Packham et al., 1998).

The Canadian province of Prince Edward Island (PEI) consists mainly of agricultural and forested land where coyotes and foxes co-occupy habitat throughout the island. It is estimated that the density of foxes and coyotes in the province is 1 and 0.4 animals / km^2, respectively (R. Dibblee, pers. comm.). In 1998, the estimated herd-level seroprevalence

of *N. caninum* on dairy farms was 63% (Keefe and VanLeeuwen, 2000). In 2005, approximately 10% of dairy farms on PEI had an estimated within-herd seroprevalence of ≥15% (Wapenaar et al., 2006a, submitted). Due to the relatively high density and close proximity of foxes, coyotes and cattle in the province, the two canid species are sighted close to farms and have access to carcasses and placentas. *Neospora*-like oocysts were observed in feces from 2 foxes (*Vulpes vulpes*) and 2 coyotes (*Canis latrans*) on PEI (Wapenaar et al., 2006b, in press). Therefore, these two wild canid populations on PEI likely are reservoirs of *N. caninum*, but their seroprevalence levels have never been investigated.

The objectives of this study were 1) to evaluate the performance of an ELISA on reference sera from foxes (both hemolysed and non-hemolysed samples) and coyotes and 2) to estimate the seroprevalence of *N. caninum* in trapped and hunted foxes and coyotes on Prince Edward Island.

6.2. Materials and methods

6.2.1. Reference samples

Reference sera from 10 *N. caninum*-negative coyotes (*Canis latrans*) were received from Dr. William Pitt (Hilo, HI, USA) and Dr. Nohra Mateus-Pinilla (Urbana, IL, USA). Sera from 6 *N. caninum*-positive coyotes were also provided by Dr. Pita Gondim (Salvador, Brazil), consisting of 4 experimentally infected coyotes and 2 field sera. The field sera were determined *N. caninum*-positive by IFAT in a previous study (Gondim et al., 2004b).

Ten ranched silver foxes (*Vulpes vulpes*) from the Canadian Centre for Fur Animal

Research at the Nova Scotia Agricultural College in Truro, Nova Scotia, were used to obtain *N. caninum*-negative and *N. caninum*-positive reference sera. After primary blood collection, all 10 foxes were vaccinated with a commercially available bovine *N. caninum*-vaccine (Neoguard®, Intervet, Whitby, ON, Canada). Five animals were given a booster vaccination in week 3. Blood was collected again from all 10 animals at 6 weeks after initial vaccination.

In order to determine whether there was a difference in test performance of the ELISA with hemolysed and non-hemolysed serum samples, all blood samples taken from these 10 foxes were split into hemolysed and non-hemolysed vials through the following process. The original vacutainer tubes with approximately 10 ml of blood were centrifuged for 10 min at 3000 × g within 24 h after collection. One ml of non-hemolysed serum was transferred into a new collection tube. The vacutainer tubes with the remaining blood were subsequently vortexed for 20 s and stored at room temperature for 24 h. Then, 1 ml of hemolyzed serum was transferred into a new collection tube. All samples were stored at -20 ºC until testing, to ensure hemolysis and preservation of antibodies. Through this procedure, 4 reference sera were obtained from each fox; a hemolysed and non-hemolysed pre-vaccination sample (negative controls), and a hemolysed and non-hemolysed post-vaccination sample (positive controls).

6.2.2. Field samples

Thirty-two registered fox and coyote trappers and hunters from across PEI were contacted and agreed to participate in this study. Fox and coyote carcasses were obtained via the hunters and trappers during their normal hunting and trapping activities from 19

October, 2004 until 24 March, 2005. No foxes or coyotes were killed deliberately for this study.

Approximately 10 ml of blood was collected from the heart or femoral artery of each carcass into a vacutainer tube. All carcasses were sampled as soon as possible after death, varying from a few hours to 5 days. Most carcasses were sampled within 24 h after death at the location where they were skinned and stored. Within 24 h of collection, blood samples were centrifuged at 1000 × *g* for 10 minutes. Based on visual assessment, the serum sample was recorded as 'non-hemolysed' or 'hemolysed'. The serum samples were stored at -20ºC until all samples were obtained.

The canine tooth of the lower jaw was also collected and sex was recorded for each fox and coyote carcass. To differentiate between a juvenile (<12 months) and an adult animal, radiographs were taken of the individual canine teeth to assess the width of the pulp cavity. The age of adult animals was determined by counts of the annual growth zones in the canine tooth cementum (Johnston Biotech, Sarnia, Ontario, Canada) (Johnston et al., 1999; Grue and Jensen, 1976).

6.2.3. Laboratory analyses

The reference and field sera were tested with an indirect ELISA (Biovet Inc., St. Hyacinthe, Canada), commercially available for use in bovine sera, according to the manufacturer's recommendations (Paré et al., 1995), but with a few modifications involving the conjugate and positive and negative control samples. As conjugate, rabbit anti-dog IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, USA) was used (dilution 1:5000) instead of the bovine conjugate provided with the kit. Pooled serum

samples from each of the coyote and fox reference sera were utilized for the creation of positive and negative controls for each species-specific ELISA-plate.

A serum dilution of 1:200 was found to be the optimal dilution to discriminate positive and negative samples, as determined by serial dilution experiments (data not shown). Serum samples were tested in duplicate and the optical density (OD) was measured in a microplate spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA, USA) at a wavelength of 405 nm. The results are reported as a sample-to-positive ratio (S/P-ratio; (average sample OD -blank OD)/(positive OD - blank OD)).

As a comparison to the ELISA, an IFAT (1:100 serum-dilution) was performed on the same coyote and fox field sera at the U.S. Department of Agriculture, Animal Parasitic Diseases Laboratory (Beltsville, MD, USA), as described by Dubey et al. (1996).

6.2.4. Statistical analyses

For test validation, likelihood ratio and two-graph receiver operating characteristic (TG-ROC) analyses were performed to determine the optimal cut-off value and sensitivity (Se) and specificity (Sp) of the ELISA for the reference fox (hemolysed and non-hemolysed) and coyote serum samples, respectively.

On the field samples, prevalence-adjusted bias-adjusted kappa (PABAK) was calculated to determine agreement (beyond that due to chance) between the ELISA and IFAT (Byrt et al., 1993). Positive and negative agreements were calculated to assess in more detail where disagreements among test results occurred (Cicchetti et al., 1990). Stata version 8 (Stata Corporation, College Station, TX, USA) was used for the statistical analyses of the data.

6.3. Results

6.3.1. Test validation using reference samples

Good discrimination between the *N. caninum*-positive and *N. caninum*-negative reference samples was observed for coyotes and for foxes in both the hemolysed and non-hemolysed form (Fig. 6.1). The S/P ratios of *N. caninum*-positive reference samples from the coyotes (n=6) ranged from 0.48 to 1.41, with a mean value of 1.00 (median = 1.08). The S/P ratios of *N. caninum*-negative reference samples from the coyotes (n=10) ranged from 0.02 to 0.36, with a mean value of 0.11(median = 0.07).

The S/P ratios of *N. caninum*-positive non-hemolysed reference samples from the foxes (n=10) ranged from 0.20 to 1.03, with a mean value of 0.73 (median = 0.82). The S/P ratios of *N. caninum*-negative non-hemolysed reference samples from the foxes (n=10) ranged from 0.00 to 0.10, with a mean value of 0.03 (median = 0.02). For the hemolysed *N. caninum*-positive fox sera (n=10), the S/P ratio ranged from 0.17 to 0.92, with a mean value of 0.60 (median = 0.67). The hemolysed *N. caninum*-negative fox sera (n=10) had S/P ratios ranging from 0.00 to 0.31, with a mean value of 0.08 (median = 0.03).

For coyotes, the likelihood ratio analysis determined the optimal cut-off value to be ≥0.40, producing a Se of 1.00 (95% CI: 0.54-1.00) and Sp of 1.00 (95% CI: 0.69-1.00). A cut-off value ≥0.19 was determined to be optimal when analyzing non-hemolysed sera of foxes, producing a Se of 1.00 (95% CI: 0.69-1.00) and Sp of 1.00 (95% CI: 0.69-1.00). Using the same cut-off value of ≥0.19 when analyzing the hemolysed reference fox sera, a Se of 0.9 (95% CI: 0.56-0.98) and Sp of 0.9 (95% CI: 0.56-0.98) was observed (Fig.

6.2).

6.3.2. Field samples

Serum was collected from 472 wild canids comprised of 201 coyotes and 271 foxes. Fifty-five percent of all the sera collected were considered as 'hemolysed'. For the foxes, 133 (49%) sera were 'hemolysed', whereas 127 (63%) sera of coyotes were 'hemolysed'. Sex was recorded for 426 animals, and was roughly equally divided among both species; 92 female and 88 male coyotes, and 115 female and 131 male foxes. The age determination showed that 48% (n=89) and 58% (n=158) of the coyotes and foxes, respectively, were less than 12 months of age. Twenty-six coyotes (14%) and 18 foxes (7%) were over 5 years of age.

Results of the IFAT showed that 6 of 472 wild canids (1.3%) were positive for *N. caninum.* By species, 3 of 271 foxes (1.1%) and 3 of 201 coyotes (1.5%) tested *N. caninum*-positive with IFAT. Using the ELISA, 12 of 472 wild canids (2.5%) were classified as positive for *N. caninum*. By species, 7 of 271 foxes (2.6%) and 5 of 201 coyotes (2.5%) tested *N. caninum*-positive with the ELISA. Five out of 6 IFAT-positive animals were also *N. caninum*-positive on the ELISA. All *N. caninum*-positive coyotes were older than 3 years of age, regardless of the test used. Conversely, 4 *N. caninum*-positive foxes on ELISA were less than 1 year of age, but all 4 of these positive juveniles were negative on IFAT (Table 6.1).

Prevalence-adjusted, bias-adjusted kappa, describing the agreement beyond chance between the IFAT and ELISA, was 0.98 and 0.95 for coyotes and foxes, respectively. Positive and negative agreements were 0.40 and 0.99 for foxes, and 0.75 and 0.99 for

coyotes, respectively.

6.4. Discussion

The ELISA used in this study performed well with the reference fox and coyote sera. Because the reference animals were experimentally infected or vaccinated, the test characteristics are likely to be overestimated. *N. caninum* assays are often validated with experimental samples, because it is difficult to obtain 'true' negative field samples. Antibody responses determined by a serological assay can be significantly stronger in experimentally infected animals compared to those infected naturally (Matsushita et al., 1987). By collecting serum from foxes 6 weeks after vaccination, high antibody levels can be expected, especially in the animals that received a second booster vaccination. In the field, animals may have been infected with *N. caninum* several years before blood was collected and subsequently their titer may be low at the time of measurement. It has been suggested that in addition to IFAT and ELISA techniques, other tests should be performed, such as immunoblotting, because of a potential confounding factor *Hammondia heydorni*, the closest phylogenetically related protozoan parasite to *N. caninum* (Gondim, 2006; Staubli et al., 2006).

Although this ELISA uses sonicated tachyzoites from bovine origin as antigen, the test performed well with sera from foxes and coyotes in the same serum dilution as recommended for bovine serum. The S/P-ratios of negative control sera were low and the S/P-ratios in the positive control sera were significantly higher, suggesting that the conjugate containing anti-dog IgG performed well by adhering specifically to antibodies from foxes and coyotes. The high S/P-ratio of positive reference coyote sera results in a

high cut-off value for this species. However, this cut-off value was based on only 16 coyote control sera, and therefore more control sera, especially *N. caninum*-positive sera, would be valuable to give a more precise estimate of Se and Sp at this cut-off value.

The TG-ROC plots are used to visually assess test characteristics at the 2 cut-off points determined for coyotes and foxes, and they show the perfect Se and Sp for non-hemolysed fox sera and coyote sera at the optimal cut-off values of 0.19 and 0.40, respectively. For cattle, cut-off S/P-ratios of 0.45 and 0.60 are recommended, with a 'suspicious' range between these values. Our optimal cut-off S/P-ratios of 0.19 and 0.40 are lower than for cattle, suggesting a weaker antibody response compared to cattle. However, this assumed weaker antibody response may be due to the lower affinity of the anti-canine antibody used as conjugate to the coyote and fox antibodies. The fact that coyotes had higher antibody titers than foxes supports this, since the coyote (*Canis latrans*) is genetically much more closely related to the dog than the foxes (*Vulpes vulpes*). Furthermore, the lower S/P-ratio observed may also be due to the high OD-value obtained from the strong positive pooled control sample that was used in each ELISA-plate.

There was a good negative agreement between the IFAT and ELISA. However, positive agreement was moderate for foxes (0.40) and coyotes (0.75), although only a small number of samples were available to compute the positive agreement estimates. IFAT is often used as the gold standard when comparing *N. caninum* serological assays. The IFAT utilizes a surface antigen and the degree of fluorescence needs to be assessed by an experienced reader, which may explain the different results obtained. The sonicated tachyzoite utilized as antigen in the ELISA may be more sensitive than whole tachyzoites

used in the IFAT because there is exposure of both internal and surface antigens. However, due to possibly identical antigens presented, a decreased Sp is expected for the ELISA because there is a greater possibility for cross-reactivity with other protozoa, such as *Hammondia* spp.. In summary, the combination of a high Se and Sp of the ELISA and the moderate to good agreement with the IFAT gives confidence that the ELISA performed adequately. Based on the Se and Sp of the ELISA being 100%, as found in this study, the estimated true seroprevalences of infection would not be different than the estimated apparent seroprevalences of infection in these wild canid populations.

It was interesting to note that all but 4 *N. caninum*-positive animals were 2 years of age and older. In fact, all of the *N. caninum*-positive coyotes were 4 years of age or older. This was in contrast with the general age structure in the collected population where approximately 50% of the animals were under 1 year of age. However, the age distribution of the trapped animals may not reflect the true age distribution of the wild population; young animals are more likely to be successfully trapped or hunted than older animals. Therefore, the calculated prevalences may be underestimated because there was a trend of increased seropositivity with age. The *N. caninum*-seroprevalence in juveniles was 1.6% (95% CI: 0.4-4.1), and the seroprevalence in animals older than one year was 4.3% (95% CI: 2.0-8.1). Based on these age-stratified seroprevalences, horizontal transmission is likely to be the primary source of infection in these wild canid populations on PEI.

Although the observed seroprevalence was low, the present study documents the occurrence of antibodies to *N. caninum* in wild canids on PEI. Low seroprevalence (0-2%) has previously been reported among red foxes in the UK (Hamilton et al., 2005),

Ireland (Wolfe et al., 2001), Sweden (Jakubek et al., 2001) and Austria (Wanha et al., 2005). The serologic evidence of *N. caninum* infection in the foxes and coyotes, poses questions about the relative importance of transmission of *N. caninum* between wildlife and livestock. The transmission of *N. caninum* has been confirmed in white-tailed deer (Vianna et al., 2005). Deer are not present on PEI, but the high density of cattle farms may represent a possible source. Cattle producers often discard bovine material on their premises, providing an easy access for food for the scavenging foxes and coyotes. Reasonable control measures may help prevent transmission of *N. caninum* to domestic ruminants, such as protection of cattle-feed from contamination with canine feces and appropriate disposal of dead livestock to avoid consumption by a definitive host.

6.5. References

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Table 6.1. Descriptive data of 13 *Neospora caninum*-seropositive animals out of 472 wild canids tested on Prince Edward Island.

Species ID	Sex	Age (years)	IFAT[a]	ELISA[b]
Coyote-1	♀	4	+	+ (0.46)
Coyote-2	♂	5	-	+ (0.61)
Coyote-3	♀	5	-	+ (0.71)
Coyote-4	♂	7	+	+ (0.91)
Coyote-5	♀	14	+	+ (1.61)
Fox-1	♂	3	+	- (0.13)
Fox-2	♂	1	-	+ (0.21)
Fox-3	♂	1	-	+ (0.22)
Fox-4	♂	1	-	+ (0.27)
Fox-5	♀	3	-	+ (0.27)
Fox-6	♀	6	+	+ (0.27)
Fox-7	♀	1	-	+ (0.31)
Fox-8	♀	2	+	+ (0.56)

[a] + = positive fluorescence of periphery of tachyzoite, serum dilution 1:100

[b] + = S/P-ratio ≥0.40 for coyotes, S/P-ratio ≥0.19 for foxes, S/P-ratio value given in brackets

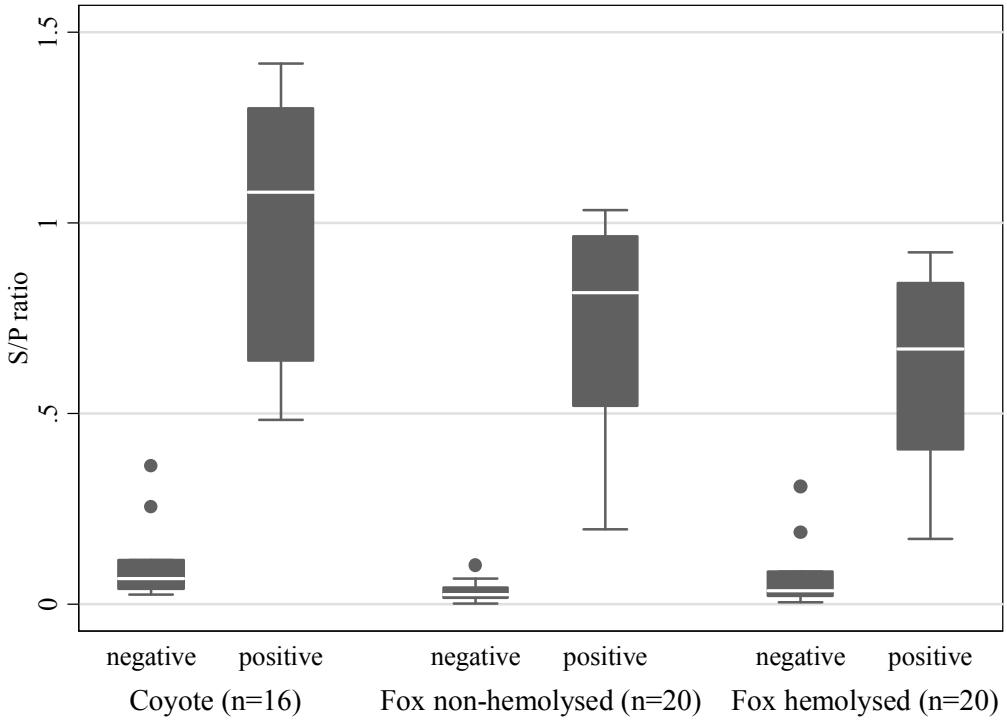

Figure 6.1. Box and whiskers plot of *Neospora caninum* reference sera from coyote sera (n=16), non-hemolysed fox sera (n=20), and hemolysed fox sera (n=20) on Prince Edward Island. The horizontal white line in the box indicates the median, the box indicates the 25th and 75th percentile, and the whiskers indicates the upper and lower adjacent values. Dots represent outside values (Tukey, 1977).

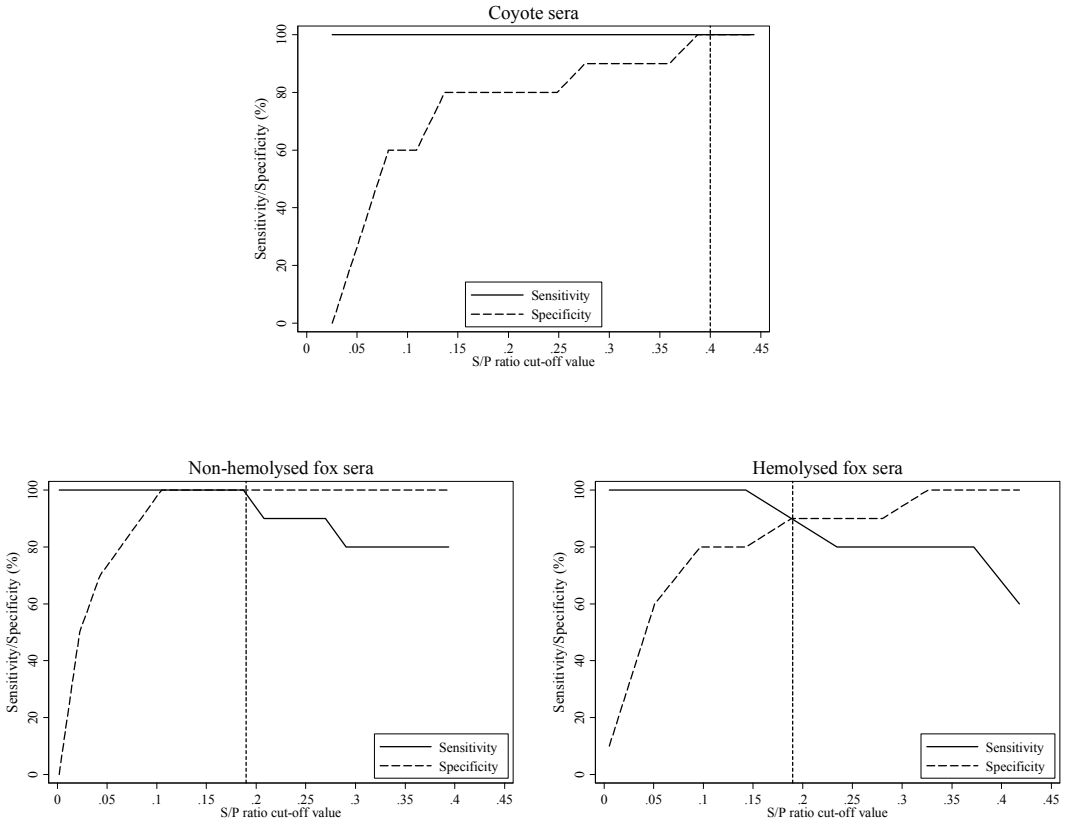

Figure 6.2. Two-graph receiver operating characteristic (TG-ROC) plots of *Neospora caninum* ELISA for coyote sera (n=16), non-hemolysed fox sera (n=20) and hemolysed fox sera (n=20) on Prince Edward Island. Vertical lines indicate the cut-off values of 0.40 for coyotes and 0.19 for foxes.

7. *NEOSPORA CANINUM*-LIKE OOCYSTS OBSERVED IN FECES OF FREE-RANGING FOXES (*VULPES VULPES)* AND COYOTES (*CANIS LATRANS*)

7.1. Introduction

Neosporosis, caused by the coccidian parasite *Neospora caninum*, is a frequent cause of bovine abortion worldwide (Dubey, 2003). The domestic dog was identified as a definitive host for the parasite in 1998 (McAllister et al., 1998). Epidemiological evidence has suggested wild canids, specifically foxes and coyotes, may also play a role in the epidemiology of bovine neosporosis. Spatial associations have been found among the density of cattle, seropositivity for *N. caninum,* and abundance of coyotes (*Canis latrans*) and grey foxes (*Urocyon cinereoargenteus*) (Barling et al., 2000). In addition, there is some evidence for a sylvatic life cycle. *Neospora caninum* was isolated from a naturally infected white tailed deer (*Odocoileus virginianus*) (Vianna et al., 2005) and a fatal case of neosporosis in a 2 months old black-tailed deer fawn (*Odocoileus hemionus colombianus*) was observed (Woods et al., 1994). One dog was shedding *N. caninum* oocysts after being fed brains of naturally infected white-tailed deer (*Odocoileus virginianus*) (Gondim et al., 2004a). In 2004, the coyote was confirmed experimentally as a definitive host for *N. caninum*, providing the most convincing evidence thus far that wild canids may play a role in the epidemiology of bovine neosporosis (Gondim et al., 2004b). However, despite the recent finding that coyotes can act as a definitive host for *N. caninum*, the occurrence of *N. caninum* oocysts in feces of wild canids in nature has yet to be reported. In addition, while red foxes (*Vulpes vulpes*) are a natural intermediate host for *N. caninum* (Almeria et al., 2002), no evidence for red foxes being a definitive

host for *N. caninum* has been reported (Schares et al., 2002). The Canadian province of Prince Edward Island (PEI) consists mainly of agricultural and forested land. The coyote population on PEI has increased rapidly since it was first encountered in 1983 and occurs throughout the island. The red fox is also very common in the province, and many foxes have lost their fear of humans as they are often fed on campgrounds and in urban areas. In addition, the estimated herd prevalence of *N. caninum* on dairy farms on PEI is 63% (Keefe and VanLeeuwen, 2000). Due to the density of foxes, coyotes, and cattle in the province, foxes and coyotes are sighted close to farms and have access to carcasses and placentas. The aim of this study was to examine the feces of free-ranging foxes and coyotes for the presence of *N. caninum* oocysts to gain a better understanding of their role in the epidemiology of bovine neosporosis.

7.2. Materials and methods

7.2.1. Sample collection

Registered trappers and hunters harvest foxes and coyotes on PEI primarily for their furs. Thirty-two trappers and hunters were contacted and agreed to participate in this study. No foxes or coyotes were killed deliberately for this study. Fox and coyote carcasses were collected from 19 October, 2004 until 24 March, 2005. All carcasses were sampled as soon as possible after each reported death, varying from a few hours to 5 days. Most carcasses were sampled within 24 hr at the location where they were stored and skinned. Fecal samples were extruded from the rectum using a disposable latex glove and stored at 4 $^{\circ}$C in individually labeled jars. If no feces were readily available at the distal end of the rectum, the rectum was stripped manually to collect a minimum of 5 g of

feces. Additionally, the canine tooth of the lower jaw and a blood sample from the heart or femoral artery were collected. Sex, date, and location where the animal was killed were recorded. Location was recorded on a map using community names. Latitude and longitude of these locations were recorded using an interactive mapping system (http://www.gov.pe.ca/mapguide). To differentiate between a juvenile (<12 months of age) and an adult animal, radiographs were taken of individual canine teeth to assess the width of the pulp cavity. The age of adult animals was determined by counts of the annual growth zones in canine tooth cementum performed at Johnston Biotech, Sarnia, Ontario, Canada (Grue and Jensen 1976; Johnston et al., 1999).

7.2.2. Fecal examination

Fecal examination was performed within 48 h after sample collection using a standard sucrose flotation method as described by Gondim et al. (2002), with slight modifications. Approximately 5 g of feces were used for the fecal flotation, with the remainder of the sample set aside for molecular analysis. Slides were examined for the presence of coccidian oocysts matching the morphologic and morphometric characteristics of *N. caninum* (Gondim et al., 2004b), and the number of oocysts on each slide was recorded.

7.2.3. Serology

Sera of foxes and coyotes from which *N. caninum*-like oocysts were identified were tested for the presence of *N. caninum* antibodies by an indirect fluorescent antibody test (IFAT) (Dubey et al., 1988) performed at the U.S. Department of Agriculture, Animal Parasitic Diseases Laboratory in Beltsville, Maryland, using a 1:50 and 1:100 dilution for

each sample.

7.2.4. Molecular techniques (PCR, cloning, sequencing)

DNA was extracted from fecal samples in which *N. caninum*-like oocysts had been observed, and from 4 fecal samples (2 foxes and 2 coyotes) with no observed *N. caninum*-like oocysts as negative controls, using a commercially available DNA extraction kit (QIAamp DNA Stool Mini Kit, Mississauga, Ontario, Canada), according to the manufacturer's protocol. The amount of fecal sample used for DNA extraction was approximately 5 g, with no purification or concentration of oocysts performed before DNA extraction. Following DNA extraction, samples were shipped by air to the U.S. Department of Agriculture, Animal Parasitic Diseases Laboratory in Beltsville, Maryland for molecular analysis. The genomic Nc5 region was selected as the target sequence for DNA amplification by nested PCR using the *N. caninum* specific primer pair Np21/Np6 (Yamage et al., 1996) in the primary reaction, followed by amplification with internal primers Np7/Np10. The Np7 primer sequence was 5' GGGTGAACCGAGGGAGTTG 3' and the Np10 primer sequence was 5' TCGTCCGCTTGCTCCCTATGAAT 3', and these primers amplified a 197 bp sequence internal to the 337 bp Np6/Np21 PCR product. To ensure that the nested Nc5 PCR assay was specific for *N. caninum*, amplifications with Np21/Np6 followed by Np7/Np10 were also performed on *H. heydorni* DNA. In addition, two different PCRs utilizing primers HhAP7F, HhAP7R and HhAP10F, HhAP10R (Sreekumar et al., 2003) specific for *H. heydorni* were conducted on DNA extracted from fecal samples containing *N. caninum*-like oocysts, as well as from fecal samples containing slightly larger oocysts (measuring 12-14μm) than what has been

described for *N. caninum*. A positive *N. caninum* control sample was DNA extracted from the brain tissue of *N. caninum*-infected mice. A positive *H. heydorni* control sample was DNA extracted from *H. heydorni* oocysts. A no template control (diluent) and DNA extracted from 4 fecal samples (2 foxes and 2 coyotes with no *N. caninum*-like oocysts observed) were included in both the primary and nested assays. PCR products were analyzed by acrylamide gel electrophoresis as described (Liddell et al., 1999). The PCR-products were cloned using a pGEM-T Easy kit (Novagen, Madison, Wisconsin). Both strands of a minimum of 3 individual clones from at least 2 independent PCR amplifications were subjected to DNA sequencing using M13 forward and reverse primers. DNA sequencing reactions were analyzed on an ABI377 DNA sequencer using Big Dye Terminators kit (Applied Biosystems, Foster City, California). Base calling was confirmed by visual inspection of the DNA sequence electropherogram. The DNA sequences were aligned using DNA Sequencher program (GeneCodes Corporation, Ann Arbor, Michigan), and the consensus sequence was used to compare DNA sequences deposited in the GenBank™ database.

7.3. Results

7.3.1. Prevalence, serology, and descriptive information

In total, fecal samples from 271 foxes and 185 coyotes were examined. Oocysts matching the morphological characteristics of *N. caninum* were observed in the feces from a juvenile female and a 2 years old female fox (0.7%), as well as a juvenile female and a 2 years old male coyote (1.1%) (Fig. 7.1). These oocysts were unsporulated, spherical to subspherical, and measured 10 to 11μm in diameter, ranging in number from

3 to 6 oocysts per coverslip. The 2 foxes were trapped and collected on the same day at the same location. It was not known whether these foxes were related (belonging to the same family). Larger spherical to subspherical coccidian oocysts (measuring 12-14μm) were observed in one more fox and one more coyote. All 4 animals containing *N. caninum*-like oocysts were negative for *N. caninum* antibodies on serology. The fox and coyote that contained larger oocysts in their feces were also negative for *N. caninum* antibodies on serology.

7.3.2. PCR

Following amplification of DNA from foxes 1 and 2, and from coyotes 1 and 2 using the *N. caninum* nested PCR, a 197 bp product was observed (Fig. 7.2). Two PCRs using primers specific for *H. heydorni* failed to yield an amplification product from these 4 DNA samples as well as from the 2 samples with larger oocysts. The *N. caninum* PCR failed to amplify DNA extracted from the 2 samples with larger oocysts and from 4 additional fecal samples included as negative controls (data not shown). A statistically significant difference ($P<0.03$) in PCR outcome between 4 samples with *N. caninum*-like oocysts and 4 negative control fecal samples was calculated using a Fisher exact test.

7.3.3. Cloning and sequencing

DNA sequencing of at least 3 clones (6 clones from fox 1, 11 clones from fox 2, 3 clones from coyote 1, and 8 clones from coyote 2) for each isolate and alignment of Nc5 sequences revealed 95-99% similarity to the Nc5 sequence of *N. caninum*. A consistent observation was the single nucleotide gap at nt 690 of the Nc5 sequence derived from *N.*

caninum oocysts recovered from fox 1 (Fig. 7.3). Although PCR amplification of *N. caninum* oocyst DNA from fox 1 and coyote 1 revealed a single clonal population, 2 distinct populations were evident by DNA sequence analysis of 11 clones derived from fox 2 oocyst DNA and 8 clones derived from coyote 2 oocyst DNA (Fig. 7.3). Although none of the 6 sequences was identical to each other, BLAST-N similarity searching of the DNA database revealed nearly complete identity to the *N. caninum* Nc5 sequence derived from a variety of hosts. Nucleotide sequence data reported are available in the GenBank™ database under the accession numbers: DQ132435 (NcFox1), DQ132436 (NcFox2a), DQ132437 (NcFox2b), DQ132438 (NcCoyote1), DQ132439 (NcCoyote2a), DQ132440 (NcCoyote2b).

7.4. Discussion

Neospora caninum-like oocysts were observed in the feces of 2/271 (0.7%) and 2/185 (1.1%) free ranging foxes and coyotes respectively in the Canadian Province of Prince Edward Island. Molecular techniques and DNA-sequencing methods employed in this study verified similarity of these oocysts to *N. caninum*. Both the prevalence and the number of *N. caninum*-like oocysts excreted in the feces of foxes and coyotes were low. Although our understanding of *N. caninum* oocyst excretion is limited, the low prevalence of oocyst excretion and the low numbers of oocysts observed in the feces of these foxes and coyotes suggest that *N. caninum* oocyst excretion is not common among wild canids on PEI. In previous experimental infections, dogs fed tissues from *N. caninum* infected calves excreted over 10,000 oocysts per day, with a total production of up to 500,000 oocysts (Gondim et al., 2002). In contrast, only 1 of 4 coyotes fed tissues

from *N. caninum* infected calves excreted oocyts and total production over 2 days was approximately 500 oocysts (Gondim et al., 2004b). Infrequent excretion in wild canids would make it difficult to find a high prevalence of fecal positive samples when sampling a limited number of samples during a short time period. Similar epidemiology has been described for *Toxoplasma gondii* (Pena et al., 2006), where the percentage of cats shedding oocysts was much lower than cats seropositive to *T. gondii.* In general, the low numbers of oocysts shed in a sample would suggest inefficiency as a definitive host, however, excretion of *N. caninum*-oocysts is influenced by a number of variables, such as the intermediate host that was ingested (Gondim et al., 2002). In a previous experiment, foxes did not excrete oocysts after experimental infection, and only a few oocysts were shed by the positive control dogs following ingestion of goat and sheep tissues (Schares et al., 2002). In this experiment, adequacy of infectious challenge, or other experimental or field conditions, may have contributed to the lack of oocyst excretion in foxes. Further experiments are needed to study oocyst shedding by wild canids.

Although *N. caninum*-like oocysts were observed in the feces of 2 foxes, this result does not confirm foxes are a definitive host for *N. caninum.* It is possible that these foxes ingested freshly passed, unsporulated, *N. caninum* oocysts through coprophagy or predation, and no sexual reproduction of the parasite took place in these foxes. Assessing the suitability of particular animals as definitive hosts is challenging and in previous experimental infections, foxes did not excrete *N. caninum* oocysts when fed tissues of infected sheep and goats. However, the foxes used for previous experimental infection were 4 months and older, and infection was performed with a single *N. caninum* isolate obtained from a naturally infected dog (Schares et al., 2002). These factors could have

contributed to the failure of foxes to excrete *N. caninum* oocysts.

The possibility that the *N. caninum*-like oocysts observed could be a different species of coccidian, with *N. caninum*-DNA amplified by PCR coming from a recent meal, needs to be considered. Theoretically, DNA from *N. caninum*-bradyzoites could have passed through the gastrointestinal tract after ingesting an infected intermediate host, excreted in the feces, and be detected by PCR. Recently, unsporulated *T. gondii* oocysts were observed in the feces of 2 dogs, probably due to coprophagy, since *Felid* spp., e.g. cats, are the only known definitive hosts for *T. gondii* (Schares et al., 2005). Although foxes prey primarily upon rabbits, rodents, birds, and invertebrates, they also scavenge carcasses and consume vegetation which could act as possible sources for *N. caninum* oocysts (Richards et al., 1995). Due to the limited amount of DNA, it was not possible to perform a PCR to detect DNA from *T. gondii*. However, the Nc 5 region used is known to be specific for *N. caninum* (Kaufmann et al., 1996).

In this study, both foxes and coyotes that excreted *N. caninum* oocysts were negative on serology. This is in agreement with previous studies where dogs did not seroconvert when shedding oocysts (Lindsay et al., 1999; Dijkstra et al., 2001).

As *N. caninum* oocysts are morphologically similar to *H. heydorni* oocysts (Schares et al., 2005), for which foxes are a definitive host (Dubey et al., 2002), molecular confirmation of oocyst identity was carried out. Ideally, a bioassay to confirm that the oocysts observed in these foxes and coyotes were *N. caninum* would have been appropriate, but it was not feasible due to the low number of oocysts observed. However, the use of PCR assays and sequencing as described herein can specifically identify *N. caninum* oocysts (Hill et al., 2001) and was used previously instead of a bioassay to

confirm *N. caninum*-oocyst shedding in coyotes (Gondim et al., 2004b). A PCR utilizing specific primers for *H. heydorni* failed to yield an amplification product, providing additional evidence that the oocysts observed microscopically were *N. caninum*. However, PCR used to identify *H. heydorni* was not a nested PCR, therefore negative *H. heydorni* results may have been caused by using a less sensitive protocol compared to that used for *N. caninum*. The genetic variation observed in the Nc5 sequence is consistent with that observed by others and may be due to the repetitive nature of Nc5 in the *N. caninum* genome (Muller et al., 2001). The reason for this variation to be present in oocysts recovered from one host (fox 2 or coyote 2) and not another (fox 1 or coyote 1) is unknown. It is unlikely due to mutation during PCR amplification of oocyst DNA because sequence analysis was performed on numerous clones derived from 2 different PCR amplifications. Genetic and biological variation among different isolates of *N. caninum* has been reported (Atkinson et al., 1999; Gondim et al., 2004c; Schock et al., 2001). The Nc5 region must be regarded as an anonymous sequence because translation of the sequence revealed a few short open reading frames. However, none demonstrated any significant homology to peptide sequences (Kaufmann et al., 1996).

Although other explanations for finding *N. caninum* DNA in fecal material must be considered, the most likely explanation is that foxes and coyotes are definitive hosts and were passing *N. caninum* oocysts formed after ingesting infected material. Despite the fact that epidemiological evidence indicates foxes and coyotes are associated with *N. caninum* infections in cattle (Barling et al., 2000), *N. caninum*-like oocysts were detected in very few foxes and coyotes on PEI and oocysts were observed in very low numbers. Further studies are required to better understand the dynamics of *N. caninum* oocyst

excretion in definitive hosts and to determine their role in the epidemiology of bovine neosporosis.

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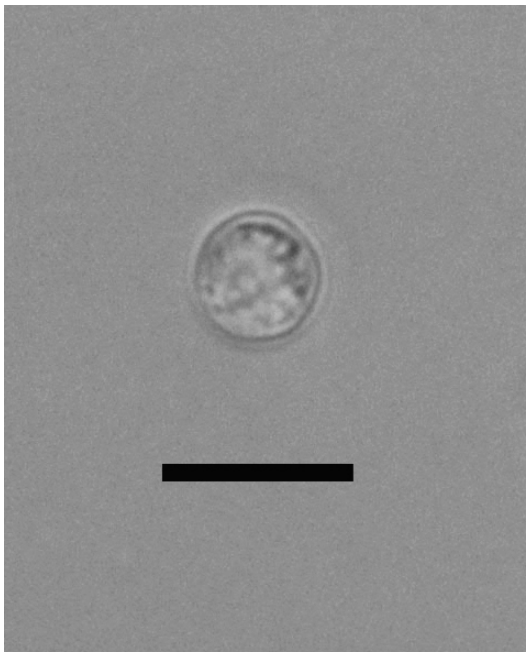

Figure 7.1. Unsporulated *Neospora caninum* oocyst observed in coyote 1 on Prince Edward Island, Canada. Bar measures 14 µm.

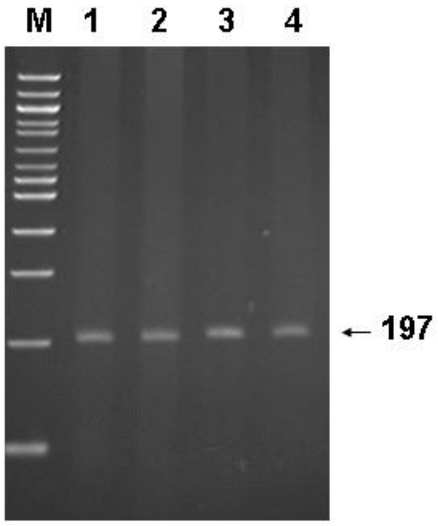

Figure 7.2. Acrylamide gel electrophoresis of amplification products from nested Np7/Np10 PCR of oocyst DNA extracted from *Neospora caninum* oocysts in feces of fox 1 (lane 1), fox 2 (lane 2), coyote 1 (lane 3), and coyote 2 (lane 4). Lane M: 100 bp molecular size markers.

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           550
Nc5        GGGTGAACCGAGGGAGTTGGTAGCGGTGAGAGGTGGGATACGTGGTTTGT
Fox 1      GGGTGAACCGAGGGAGTTGGTAGCGGTGAGAGGTGGGATACGTGGTTTGC
Fox 2a     GGGTGAACCGAGGGAGTTGGTAGCGGTGAGAGGTGGGATACGTGGTTTGT
Fox 2b     GGGTGAACCGAGGGAGTTGGTAGCGGTGAGAGGTGGGATACGTGGTTTGT
Coyote 1   GGGTGAACCGAGGGAGTTGGTAGCGGTGAGAGGTGGGATACGTGGTTTGT
Coyote 2a  GGGTGAACCGAGGGAGTTGGTAGCGGTGAGAGGTGGGATACGTGGTTTGT
Coyote 2b  GGGTGAACCGAGGGAGTTGGTAGCGGTGAGAGGTGGGATACGTGGTTTGT
                                                           •

           600
Nc5        GGTTAGTCATTCGTCACGTTGAAATCAGCCTGCGTCAGGGTGAGGACAGT
Fox 1      GGTTAGTCATTCGTCACGTTGAAATCAGCCTGCGTCAGGGTGTGGACAGT
Fox 2a     GGTTAGTCATTCGTCACGTTGAAATCAGCCTGCGTCAGGGTGTGGATAAT
Fox 2b     GGTTAGTCATTCGTCACGTTGAAATCAGCCTGCGTCAGGGTGTGGACAGT
Coyote 1   GGTTAGTCATTCGTCACGTTGAAATGAGCCCGCGTCAGGGTGTGGACAGT
Coyote 2a  GGTTAGTCATTCGTCACGTTGAAATCAGCCTGCGTCAGGGTGAGGACAGT
Coyote 2b  GGTTAGTCATTCGTCACGTTGAAATGAGCCCGCGTCAGGGCGTGGACAGT
                                  •    •          • •   • •

           650
Nc5        GTGTCAATGATACTTATCGAGAGTTCAGTGTTCTGTGTTGAGGCAACACC
Fox 1      GTGTCAATGATACTTATCCAGAGTTCAGTGTTCTGTGTTG:TGCAACACC
Fox 2a     GTGTCAATGATACTTATCCAGAGTTCAGTGTTCTGTGTTGAGGCAACACC
Fox 2b     GTGTCAATGATACTTATCGAGAGTTCAGTGTTCTGTGTTGAGGCAACACC
Coyote 1   GTGTCAATGATACTTATCGAGAGTTCAGTGTTCTGTGTTGAGGCAACACC
Coyote 2a  GTGTCAATGATACTTATCCAGAGTTCAGTGTTCTGTGTTGAGGCAACATC
Coyote 2b  GTGTCAATAATAGTTATCGAGAGTTTAGTGTTCTGTGTTGAGGCAACACC
                  •          •      •               ••      •

           700
Nc5        GGCGGCACTGATGACGGGGGAGATTATTCATAGGGAGCAAGCGGACGA
Fox 1      GGCGGCACTGATGGCGGGGGAGATTATTCATAGGGAGCAAGCGGACGA
Fox 2a     GGCGGCACTGATGACGGGGGAGATTATTCATAGGGAGCAAGCGGACGA
Fox 2b     GGCGGCACTGATGACAGGGGAGATTATTCATAGGGAGCAAGCGGACGA
Coyote 1   GGCGGCACTGATGACGGGGGAGATTATTCATAGGGAGCAAGCGGACGA
Coyote 2a  GGCGGCACTGATGACGGGGGAGATTATTCATAGGGAGCAAGCGGACGA
Coyote 2b  GGCGGCACTGATGACGGGGGAGATTATTCATAGGGAGCAAGCGGACGA
                       • •
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Figure 7.3. Alignment of Np7/Np10 sequences derived from nested PCR amplification of DNA from the *Neospora caninum* Nc-1 strain Nc5 sequence and *Neospora caninum* oocysts in feces of fox 1, fox 2 (a & b), coyote 1, and coyote 2 (a & b). The symbol • and bold underlined nucleotides indicate a nucleotide difference between at least one of the fox or coyote Np7/Np10 sequences and the *N. caninum* Nc-1 strain Nc5 sequence as reported by Yamage et al. (1996).

8. GENERAL DISCUSSION

8.1. Introduction

The main aim of the studies described in this thesis was to obtain more epidemiological information on *N. caninum* on PEI and the purported sylvatic life cycle of *N. caninum*. Certain topics were studied in detail, such as the shedding of *Neospora*-like oocysts in fox and coyote feces (Chapter 7), and the effect of hemolysis on serological assays (Chapter 4). Evaluation and validation of diagnostic tests for dairy herds and wild canids (Chapter 2, 3, 6) were required. Validated tests could then be applied to characterize herd status and to examine the status of wild canids. To further explore the presumed role of wild canids in the *N. caninum* epidemiology, Chapter 5 describes the population structure and habitat characteristics of wild canids on PEI. The results of these studies are discussed in relation to the literature. Lastly, suggestions for further research are made.

8.2. Prevention and control of *N. caninum*

From the results of this thesis and other research it seems that prospects of controlling the presence of *N. caninum* infection in Canada are favourable. Although the herd prevalence is reported to be high (Haddad, 2006), in Chapter 2 is described that the within-herd prevalence is below 15% in most dairy herds. With the appropriate management measures the risk of spread of the parasite within and between herds can be minimized.

The dairy industry in The Netherlands and Canada has strong similarities; more than 80% of dairy farms participate in a dairy herd improvement program that carries out monthly testing of dairy herds, and collects a variety of information on all lactating cows with regard to milk production. Within and between herd *N. caninum* prevalences are similar in both countries; however, several risk factors are different. In The Netherlands, the density of dairy farms and domestic dogs is higher and the climate is more moderate. Furthermore, except for foxes, wild canids, such as coyotes, are not present. Consequently, a similar control program as in The Netherlands could be implemented in Canada, with adjustments to specific risk factors identified for *N. caninum* in Canada.

In The Netherlands, a voluntary control program has been in place since 2004, where bulk milk is tested three times yearly by the Dutch Animal Health Service in Deventer. When results are favourable (suggesting *N. caninum* is not detected in the herd) once a year the results are presented to the producer with recommendations for their specific situation. To remain *N. caninum*-free, recommendations are provided to producers related to breeding advice, control of dogs on the property and purchase of animals. These recommendations are also communicated to the producers' herd health veterinarian. If results are unfavourable (suggesting *N. caninum* is present in the herd) recommendations are given immediately to prevent impending problems. Together with the veterinarian, individual serologic testing is used for detection of infected age-groups of cattle and strategic culling can help in reducing damage imposed by *N. caninum*. An additional advantage of this program (provided against a yearly fee of approximately 100 CA$) is that cows which have aborted are serologically tested at no extra charge. Currently, 4000 dairy farms are participating in this program, which has been well received by producers

(Dijkstra et al., 2005).

With the information collected on wild canids on PEI (Chapters 5, 6 and 7), it can be concluded that for PEI it is especially important to avoid spread via wild canids between and also within herds. More emphasis should be placed on proper disposal of carcasses, aborted foetuses, raw meat or slaughter offal from infected cows. This is an essential component to prevent infection of intermediate and definitive hosts by ingesting infected tissue. A dead stock collection and disposal program is available on PEI. Disposal of dead stock by dumping in wooded areas, burial, or improper composting is prohibited by provincial regulations. However, not all producers use this dead stock collection service. Producers may not be aware of the risks involved in dumping carcasses in wooded areas on their property. Besides *N. caninum,* other parasites transmitted by canids, such as *Sarcocystis* spp., can be detrimental to their livestock and the risk of coyotes attacking young stock or domestic cats and dogs has to be considered once canids are accustomed to find food on the farm property. However, when informed about these risks, producers will likely be motivated to use dead stock collection services that are available. By removing easy food access for foxes and coyotes, these canids will find other food sources not close to farms. In addition, farm dogs are less likely to pose a risk for horizontal transmission if *N. caninum*-infected tissue is not available on the farm. Although there is no unambiguous evidence that dogs and coyotes are a source of post natal infection in PEI dairy herds, they are confirmed to be definitive hosts (Dijkstra et al., 2001; Gondim et al., 2004; McAllister et al., 1998) and therefore should not be allowed to contaminate cattle feed. Keeping dogs away from calving pens and feed storage areas reduces the risk of horizontal transmission (Dijkstra et al., 2002).

On *N. caninum*-positive farms, individual testing may be valuable. Infection status should be given a high priority in the culling decision process. The within-herd prevalence can be reduced by making sure that test-positive heifers are not kept for replacement. Embryo-transfer can be used if valuable offspring is expected from a *N. caninum*-positive animal (Baillargeon et al., 2001). In cases where new animals are purchased, these should first be tested and declared free from *N. caninum* infection. Obviously, the measures aiming at eliminating infection in a herd should be combined with preventive measures to avoid re-infection. Because the epidemiology of *N. caninum* remains incomplete, unknown intermediate and definitive hosts may play a role in transmission of the parasite. Consequently, elimination of *N. caninum* on a farm can not be guaranteed and more research is needed to provide more information on possible other hosts involved.

8.3. Role of wild canids in the transmission of *N. caninum*

Coyotes and foxes are invading agricultural and urban areas on PEI, and the closer contact can lead to an increase in disease transmission to domestic livestock and humans. With cattle being on pasture in summer and fed freshly chopped grass or silage when inside, the opportunity for transmission of *N. caninum* to cattle via ingestion of oocysts is likely to occur. However, a low seroprevalence of *N. caninum* was observed in wild canids (Chapter 6), suggesting that wild canids do not play a major role in the *N. caninum* epidemiology on PEI. However, a previous study has shown that dogs shedding *N. caninum* oocysts can be negative by *N .caninum* serology (Dijkstra et al., 2001). The 2 foxes and 2 coyotes that were shedding *Neospora*-like oocysts in our study were sero-

negative (Chapter 7), and the 12 *N. caninum*-positive animals by ELISA were not shedding any oocysts. It is possible that the cyst stage in canids does not elicit an extended antibody response; however, no research data are available to support this assumption. Another hypothesis for the low antibody titres could be that definitive hosts when infected with *N. caninum* infected tissue, harbour the parasite until it sheds oocysts, and afterwards eliminate the parasite. The presence of parasites in the digestive tract and short contact time with the immune system via blood may cause a weak immune response. In addition, the immune response needs several weeks to develop to detectable levels. It is interesting to note that the seropositive wild canids were mainly older animals, suggesting that post-natal infection was more likely the route of infection than vertical transmission. This is different from what is observed in cattle, where vertical transmission is the most dominant transmission route (Bergeron et al., 2000; Frössling et al., 2005; Schares et al., 1998).

8.4. Diagnosis of *N. caninum* in cattle and wild canids

The serological assays that were evaluated (Chapter 3) constitute a useful set of diagnostic tools that can be readily applied in control programs and research. The use of bulk milk as diagnostic tool instead of serum may be the preferred primary tool in control programs because of the cost and convenience of sample collection. Although an increased sensitivity would be helpful, the test characteristics when using bulk milk (Chapter 2) are acceptable for applying this assay in the field. If a herd is *N. caninum*-positive and individual information is needed on *N. caninum*-status, most serological assays discussed in Chapter 3 are recommended, with exception of the *N. caninum*

agglutination test. In general, the specificity of currently available assays is good; the major difficulty is related to the sensitivity of most assays. However, this shortcoming may be more due to a lack of *N. caninum* antibodies present in chronically infected animals, than a true lack of sensitivity of the assay. The use of individual milk samples has shown promising results in several studies (Björkman et al., 1997; Chanlun et al., 2006; Milne et al., 2006; Schares et al., 2005) and may, in the future, replace serology because of the convenience of sample collection. However, no commercially available test designed specifically for milk is currently available, and the quantity of research evaluating and validating serological assays greatly exceeds those examining the use of milk as diagnostic sample for *N. caninum*, making the serological assays at this time perhaps a more reliable source of information.

An indirect ELISA was preferred over a competitive inhibition ELISA to investigate the serostatus of wild canids (Chapter 6). Although both assays performed adequately when analysing non-hemolysed sera, a difference in test performance was observed when using hemolysed sera. Interestingly, when analysing cattle sera, sample quality did not show the same variety using the same assays. The effect of hemolysis on performance of assays depends on which diagnostic assay is used and, especially when dealing with samples from wildlife, this phenomenon requires further research.

8.5. Suggestions for further research

The oocysts observed in fox and coyote feces (Chapter 7) were likely *N. caninum*, but experimental infection of foxes, similar to that successfully performed for coyotes, is needed to confirm the fox as a definitive host. An experimental infection in foxes was

conducted previously, but certain modifications could be made to increase the chances that foxes could shed *N. caninum* oocysts. For example, the age of animals and their immune status may be important factors that can be controlled in an experiment. The worldwide distribution of foxes makes this species an important potential definitive host to investigate. Besides foxes, alternative intermediate and definitive hosts should be investigated. Scarce information is available on the role of birds, raccoons and rodents in the epidemiology of *N. caninum*, however, several studies (Bartels et al., 1999; Huang et al., 2004; McGuire et al., 1999) suggest these animals may play a role in the epidemiology of this parasite and thus additional research is essential to further explore this interesting field. To confirm the transmission from cattle to wild canids in a field situation, genotyping *N. caninum* strains found in each species could further prove the hypothesized transmission.

To identify the risk of fecal material on pasture as a possible source of infection, more studies are necessary to learn about survival times of oocysts, and identify influential factors, such as temperature, humidity, and sunlight. It is not known whether oocysts survive the process of making silage or how long they remain infective on pasture. The survival time of tachyzoites in placentas after calving, and tachyzoites and bradyzoites in dead stock is also unknown.

Serology of *N. caninum* could be improved by investigating differences in antigenic response to bradyzoite and tachyzoite stages of infection. More information in this area may help to explain different results obtained by different serological techniques, such as NAT, IFAT and ELISAs. With the increased use of PCR as an important tool to determine infection status, it remains important to ensure specificity of primers,

especially when PCR is used to define new hosts, like in Chapter 7. When determining infection status of animals, PCR may not be the preferred method due to the fact that DNA of the parasite needs to be present in the small amount of sample that can be utilized with this technique.

With regard to the analysis of hemolysed samples, as described in Chapter 4, it would be beneficial to investigate in more detail the causes of the reduced test performance observed in ELISAs and to determine whether there are practical techniques which can be used to overcome this problem.

The role of immunity in cattle related to *N. caninum* and the risk of abortion is an area that is not discussed in this thesis, but it is important in the understanding of the pathogenesis. Why certain cows abort and others not, remains an area of speculation at this time. Experimental studies could provide more information, and possibly an access to control of abortions in the future.

Vaccination and treatment of *N. caninum* has so far not proven to be very successful. Vaccination has shown contradictive results in several studies, with one of the main drawbacks being that once animals are vaccinated all information on their true *N. caninum* status is lost. Efficacious vaccines to protect animals and humans against parasitic infections are few and difficult to produce, due to the antigenic variability created by the different stages of many parasites. However, research in this area is ongoing and will hopefully create possibilities for control in the future. Treatment of neosporosis is dealing with similar difficulties, some antiprotozoal drugs claim to have some effects on *N. caninum* infection, but this is probably only related to the tachyzoite stages of the parasite. However, this may prevent abortion and research in this area still

needs attention. Although treatment will likely not be an option in the food animal industry, information in this area may provide better treatment methods for other animals, such as dogs, infected with *N. caninum.*

The bulk milk ELISA could be used in future monitoring programs in its current form. However, a more detailed study investigating the impact of herd and cow level factors such as herd size, production and lactation may provide information to more accurately estimate the within-herd prevalence.

To be able to provide adequate information to producers, it is important to assess the effect of control programs. Is enough known about *N. caninum* to control and decrease *N. caninum* prevalence and its related economic losses? May be there is an increased risk of an epidemic *N. caninum*-outbreak due to the creation of naive herds when a control program decreases the prevalence in the herd.

Providing producers with useful information about neosporosis is an essential part in controlling disease; studies directed at improving knowledge transfer and compliance of producers to recommendations from veterinarians can help in establishing a successful *N. caninum* control program.

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